National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

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A validation study

RIVM report 607711015/2014

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Colophon

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Orale biobeschikbaarheid van lood uit Nederlandse stedelijke ophooglagen

In Nederland zijn de bodems van oude binnensteden (ophooglagen) in het verleden gevormd door stadsafval en puin, onder andere afkomstig van industriële activiteiten. Dit materiaal is vaak verontreinigd met lood, en zo ook de bodems. Vooral kinderen zijn gevoelig voor de schadelijke effecten van lood als zij dat via de mond binnenkrijgen. Een te hoge blootstelling aan lood kan de ontwikkeling van de hersens verstoren. Er bestaan meerdere laboratorium-modellen die schatten hoeveel lood uit de bodem in het maag-darmkanaal vrijkomt en vervolgens bij kinderen in het bloed kan terechtkomen (biobeschikbaarheid). Het RIVM heeft onderzocht hoe goed drie van deze modellen deze schatting kunnen maken. Hieruit blijkt dat al deze methoden sterke én zwakke punten hebben, maar dat het zogeheten Unified BARGE Model het meest geschikt is om biobeschikbaarheid van lood in ophooglagen te schatten.

Met de drie modellen is de biobeschikbaarheid van lood in zes bodems geschat. De uitkomsten zijn vervolgens vergeleken met de resultaten van biobeschik-baarheidsonderzoek met jonge varkens. De manier waarop lood zich in het maag-darmkanaal van deze dieren gedraagt, is vergelijkbaar met het gedrag in dat van kinderen. Het Unified BARGE Model en het Tiny-TIM model laten eenzelfde patroon zien als de dierproeven, maar de uitkomsten van Tiny-TIM leiden tot een onderschatting van de werkelijke biobeschikbaarheid. Het IVD-model blijkt alleen geschikt als wordt gecorrigeerd voor het kalkgehalte in de bodem. Een relatief eenvoudige methode om de hoeveelheid beschikbaar lood in een bodem te schatten is extractie met verdund salpeterzuur. Deze methode kan als een eerste screening worden gebruikt om de hoeveelheid biobeschikbaar lood in een bodem te schatten.

Uit de resultaten van de dierproeven kan een standaardwaarde voor de biobeschikbaarheid van lood in stedelijke ophooglagen worden afgeleid. Beleidsmakers kunnen deze waarde als maatstaf gebruiken om te bepalen hoeveel lood beschikbaar is om door het menselijk lichaam te kunnen worden opgenomen. Op basis van de standaardwaarde en het totaalgehalte aan lood in de bodem wordt bepaald of er een onacceptabel risico voor de gezondheid is en maatregelen nodig zijn. Het gebruik van deze standaardwaarde heeft als voordeel dat er geen experimenten met de testmodellen nodig zijn, wat geld en tijd bespaart. De bevindingen van dit onderzoek geven aan dat er meer lood in de bodem beschikbaar is dan eerder werd verondersteld. Dit kan aanleiding zijn om de normstelling van lood in bodem opnieuw te bekijken.

Trefwoorden: lood, biobeschikbaarheid, bioaccessibility, bodem, in vitro digestie, in vivo, validatie

Abstract

Oral bioavailability of lead from Dutch made grounds

The soils of historical inner cities in the Netherlands (made grounds) are often contaminated with lead as result of their formation by dumped trash and debris from industrial activities in the past. Children are particularly susceptible to the adverse effects of lead ingestion; a high exposure to lead can affect brain development. Several *in vitro* laboratory models exist which can estimate the amount of lead released from the soil and able to enter a child's blood (bioavailability). The RIVM has examined the predictability of three of these models. All models have their strengths and limitations, but the so-called Unified BARGE model appears to be the best applicable model for estimating the bioavailability of lead in made grounds.

The bioavailibility of lead in six soils was estimated using the three models and the results were compared with the results of a bioavailability study conducted on juvenile swine. The behavior of lead in the gastrointestinal tract of swine was comparable to that in children. Both the Unified BARGE model and the Tiny-TIM model show the same pattern as the results of the animal experiments. However, the Tiny-TIM values underestimate the true bioavailability. The IVD model is only suitable after a correction for calcium content of the soil. An alternative, relatively simple method is to estimate the bioavailable lead in a soil using an extraction with diluted nitric acid. This method can be used as a screening method to estimate the bioavailability of lead in the soil.

From the results of the swine study, a standard value can be derived for the bioavailability of lead in made grounds. Policy makers can use this value as a benchmark to determine which fraction of the lead is bioavailable for uptake in the human body. Consequently, by combining the total lead content in the soil and the standard value, it can be determined whether there is a health risk and whether measures should be taken. The use of this standard value renders experiments with laboratory models redundant, thus saving time and money. The outcome of this study indicates that more lead is bioavailable than previously assumed. This may be a reason to re-evaluate the criteria for lead in soils.

Key words: lead, bioavailability, bioaccessibility, soil, in vitro digestion, in vivo, validation

Contents

Summary

Children in historical inner cities may be exposed to lead because the soil in these areas, called 'made grounds', holds lead-containing waste products and building rubble associated with centuries of urban development and industrial activities. In 2009, the bioaccessibility of lead in Dutch made grounds was measured with two *in vitro* digestion models potentially suitable for human risk assessment. These models generated very different results (factor 5 difference).

The aim of the present study was to validate three *in vitro* digestion models with respect to the bioavailability of lead from Dutch made grounds. The bioavailability of lead from six made ground samples was measured in an *in vivo* juvenile swine study (model for toddlers). Homogenized soil samples for six contaminated sites were sieved at 2 mm. The total amount of lead was determined with X-ray fluorescence and extraction was carried out with *aqua regia.* Additionally, an extraction with 0.43 M HNO_3 was conducted representing the potentially available fraction. Soil was added to a small portion of moist pig feed and fed to groups of six juvenile male pigs once a day for one week. The lead exposure period was followed by an elimination phase (no lead exposure) of one week. Blood samples were collected (almost) daily during the exposure and elimination phase in order to measure lead concentration. After sacrifice, livers were extracted and analysed for lead. The *in vivo* results were compared to the bioaccessibility values of the same soils measured in the three *in vitro* digestion models.
The *in vitro* digestion models tested were the dynamic Tiny-TIM model (n=2), and the static models IVD (In Vitro Digestion model; n=4) and UBM (Unified Barge Method; n=3). For the latter method, the lead bioaccessibility was measured after the gastric phase (referred to as $UBM_{gastric}$) as well as after the intestinal phase (referred to as UBM). In order to compare the different models, in addition to lead from soils, the bioavailability and bioaccessibility of a reference substance (lead acetate) was measured.

Due to a low bioavailability of lead acetate in the *in vivo* validation study, the relative bioavailabilities (RBA, i.e. the bioavailability of lead in soil divided by the bioavailability of lead acetate) for the blood data were calculated using the bioavailability for lead acetate determined in the pilot study (13%). The absolute bioavailability of lead for children, as determined in the *in vivo* study, ranged from 22%- 45% for the six soils sampled in this study. The results of Tiny-TIM and UBM correlated well with the RBA_{blood} data, but those of Tiny-TIM underestimated the bioavailability. The bioaccessibilities determined with IVD and $UBM_{gastric}$ showed a low correlation with the *in vivo* data. A multiple regression analysis showed that when the calcite fraction of the soil was taken into account, the IVD could predict the RBA_{blood} well. However, a scientific rationale for a calcite correction is limited and the analysis was based on six soils only. Thus, application of a correction factor was not considered appropriate. At present, the UBM model is the best applicable model, although it is a fasted model not mimicking a semi-fed child and has a high solid: liquid ratio of 1:100, which is known to decrease the lead bioaccessibility. If this type of model is desirable, further model adaptations and validations are required.

The lead extractions with 0.43 M HNO_3 correlated well with the relative bioavailability measured in swine. Since these are simple and inexpensive extractions, this method is recommended as a screening method in location-specific estimates of the bioavailability of lead in made grounds. Since this method is not physiologically based and only six soils were tested, additional validation is needed to ensure that extraction with diluted nitric acid does simulate bioavailability of lead from soils, before it can be used as a reliable method rather than for screening purposes only.

Because of the small spread in physicochemical characteristics as well as in the bioavailabilities of the made grounds as measured in the *in vivo* experiment, it is appropriate to apply a generic bioavailability correction factor in human risk assessment of lead in made grounds from (historical) inner cities. Nevertheless, the results indicate that the lead from some of the Dutch made grounds may be as bioavailable as lead from food. From the data generated in this study, it can be concluded that the value of *Rel F* of 0.4, currently used as generic value, is too low from a scientific point of view. An increase of the *Rel F* to 0.58 (P50)-0.84 (P80) is recommended, depending on the level of conservatism desired by the risk manager. This range corresponds with the *Rel F* determined for 90 soils in the previous study, which equaled 0.67 (P50) – 0.91 (P80). Due to the small variation in the RBAs of the studied made grounds, the role of location-specific determination of bioaccessibility of lead in made grounds is expected to be small.

List of abbreviations

ABA	absolute bioavailability
AUC	area under the curve
BA	bioavailability
BAc	bioaccessibility
BARGE	Bioaccessibility Research Group Europe
ICP-MS	inductively coupled plasma mass spectrometry
IVD	*in vitro* digestion
Pb	lead
PbAc	lead acetate
RBA	relative bioavailability (relative to PbAc)
RBAc	relative bioaccessibility (relative to PbAc)
RSD	Relative standard deviation
TIM	TNO *in vitro* model
UBM	Unified BARGE model
XRF	X-ray fluorescence

1 Introduction

Dutch made grounds
The soil in the historical inner (city) areas of many Dutch cities and villages is often polluted with lead (Pb). This lead originates from various sources, including the accumulation of lead-containing waste products and building rubble associated with centuries of urban development and industrial activities. This has resulted in a layer referred to as (man) made ground. This lead mainly originates from white lead (lead carbonate) factories, coal combustion waste, and construction waste. The lead concentrations in soil reach levels high enough to cause a potential health risk for humans. Especially children may be exposed to too high amounts of lead by ingesting lead-containing soil after hand-to-mouth contact. In addition, lead is better absorbed in children than in adults. This notion originates from a number of studies which suggest that lead is absorbed by the same mechanism as calcium (Diamond et al., 1998). Calcium is better absorbed in children than in adults as their growth requires more calcium (Clarkson, 1993; Fullmer, 1992) The main target for lead toxicity is the nervous system (reviewed by Bellinger (2004), Koller et al. (2004), Lidsky et al. (2003) and Needleman (2004)), resulting in a reduced IQ. At high levels of exposure, lead can severely damage the brain and kidneys (Boreiko and Battersby, 2008).

In recent years, international and European health-based guidance values for lead exposure have been amended several times. In 2010, the European Food Safety Authority's (EFSA) Panel on Contaminants in the Food Chain concluded that the provisional tolerable weekly intake (PTWI) of 25 µg/kg b.w. was no longer appropriate (EFSA, 2010). The Panel decided that, as there was no evidence for a threshold for a number of critical endpoints including developmental neurotoxicity and adult nephrotoxicity, it would not be appropriate to derive a PTWI. This conclusion was confirmed by JECFA in 2010 and the PTWI was withdrawn (FAO/WHO, 2011). Using an alternative measure, the 2010 EFSA opinion identified a 95th percentile lower confidence limit of the benchmark dose of 1% extra risk (BMDL01) of 0.50 µg/kg b.w. per day for developmental neurotoxicity in young children.

In light of the particular concern regarding lead exposure in children, it is important to improve estimates of the bioavailability of lead in Dutch made grounds.

Bioaccessibility versus bioavailability
Oral bioavailability (F) can be divided into three different major processes (equation 1; Oomen et al., 2006):

$$F = F_B \times F_A \times F_H \quad [1]$$

After ingestion of soil, the total bioavailability (F) depends on the amount of contaminant released from the matrix (i.e. soil) during digestion in the gastrointestinal tract into the intestinal fluids. This is referred to as bioaccessibility (with F_B the bioaccessible fraction). Part of the bioaccessible

fraction is transported across the intestinal epithelium and reaches the portal vein (absorbed fraction; F_A). Metabolism of the contaminant may occur in the intestinal epithelium and/or in the liver. The fraction that is not metabolized (F_H) passes the liver via the portal vein to the systemic circulation and is transported throughout the body, and represents the bioavailable fraction (F). Note that lead is not metabolized, resulting in a F_H fraction of 1.

The *in vitro* models simulate the release of substances from their matrix during digestion, and can be applied to estimate the bioaccessibility ($F_{B)}$ of lead from soil. Two *in vitro* digestion models are currently in use in the Netherlands: The In Vitro Digestion (IVD) model and the Tiny TNO In Vitro Model (Tiny-TIM). A third model, the Unified BARGE method (UBM), is the digestion model used by the Bioaccessibility Research Group Europe (BARGE) and has many similarities with the IVD model.
Note that in animal models, the bioavailability (F) of a substance is investigated.

Current practice
The current practice of risk assessment of lead in soils is illustrated in Figure 1. The Dutch Intervention Value for lead in standard soil is 530 mg/kg. This standard relates to a maximum permissible risk (MPR) value of 3.6 µg/kg bw/day, which is based on epidemiological and toxicological studies (Baars et al., 2001)[1]. The studies which determined the MPR value were based on lead intake in children via food. However, the bioavailability of lead from food is often higher than the bioavailability of lead from ingested soil. Therefore, for human risk assessment of lead from soil, the reference value is corrected with the relative bioavailability correction factor (Rel F). By default, a generic value of 0.74 that applies to all grounds is used for the Rel F. This value is based on the relative bioavailability of lead in soils as determined in the In Vitro Digestion (IVD) model (Hagens et al. 2008).

When the lead concentration in soil exceeds the Intervention Value, further risk assessment is performed using the computer programme Sanscrit (www.sanscrit.nl). If the reference value is not exceeded, no further action is required unless there is a specific 'sensitive' situation, such as a vegetable garden. Sanscrit is a decision-support tool that can be used to determine if there is an unacceptable risk of soil pollution for humans and for the environment. Based on the outcome, it can be determined whether further action, such as remediation or redevelopment, is required to ensure safety. Two main aspects of the tool can be distinguished. First, the relevant human exposure scenario can be determined. Secondly, further soil-specific evaluation can be performed by adjusting the value for Rel F. For made grounds, a Rel F of 0.4 is currently used in soil policy (Circulaire bodemsanering, 2013). A site-specific Rel F can also be calculated by determining the bioavailability of lead from the soil in *in vitro* digestion models.

Based on the adjusted Rel F in the Sanscrit calculations, it can be determined if there is a potential health risk and if further action is required.

[1] The current MPR value is lowered to 2.8 µg/kg bw/day, with retention of the reference value of 530 mg/kg.

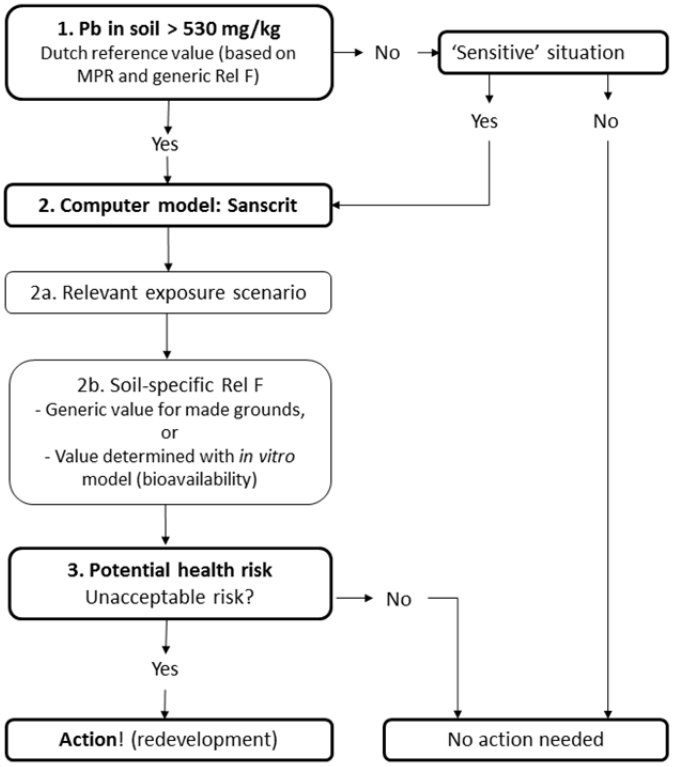

Figure 1 Current practice of risk assessment of lead

Aim and method of present study
This report describes the validation study as recommended in the workshop. The aim of the study is to select the *in vitro* model that, for children, gives the best prediction of the bioavailability of lead in made grounds. The selected model can be applied in the site-specific risk assessment of lead in Dutch made grounds as explained above (Staatscourant, 2012). Additionally, if the results from the *in vivo* experiment allow, a generic bioavailability factor (Rel F) for lead in Dutch made grounds will be deducted.

The *in vivo* study was performed in juvenile swine, which provide a good model for the gastrointestinal system of a human child (US EPA, 2007). Weis et al. (1991) and Casteel et al. (1996) determined that gastric function in juvenile swine is sufficiently similar to that of human children for juvenile swine to serve as a model for predicting RBA of soil-borne lead in children. This view is supported by several reviews on the comparative anatomy and physiology of the human and pig gastrointestinal systems (Dodds, 1982; Miller et al., 1987; Moughan et al., 1992). The swine were fed a small amount of food containing soil from made grounds. The same made ground samples were studied in the three *in vitro* models (IVD, Tiny-TIM and UBM). In addition, the samples were extracted with 0.43 M HNO_3, as this is a method used in ecological studies to

extract the potential bioavailable fraction. HNO_3 extractions of soil can be used to predict the concentration of free (unbound) metals in the soil, which is related to the bioaccessibility of the metals in the soil (Dijkstra et al., 2009; Groenenberg et al., 2010). This is in contrast with *aqua regia* which also extracts non-bioavailable parts of the metals from the soil.

Previous study
In 2009, the bioaccessibility of lead in 90 Dutch made grounds was determined with the IVD model (Hagens et al., 2009). Sixteen of these samples were also investigated with the Tiny-TIM model. The results of the two *in vitro* models appeared to be very different: the bioaccessibility of lead determined with the Tiny-TIM model was, on average, a factor five lower than that of the IVD model. In an international workshop in 2009 organized by TNO and RIVM, it was concluded that the different results of the two methods were mainly caused by the pH of the gastric phase and the different separation techniques (ultra-filtration vs. centrifugation) (Bakker, 2009). Another conclusion was that an *in vivo* validation study, preferably using young swine, would be required for a responsible justification of the application of the *in vitro* models for risk assessment of lead in soils. The model showing the highest correlation with the *in vivo* data (for a large bioavailability range) will be considered the best model to predict the bioavailability of lead in made grounds for young children (Bakker, 2009). In addition, the selected model should be: 1) simple (feasible to operate in routine application at more than one location), 2) responsive to different lead and soil characteristics, and 3) accompanied by rigorous Quality Assurance/Quality Control data requirements (e.g. with regard to recoveries, blanks, reproducibility) (Bakker, 2009).

Use of reference substance
To compare the bioaccessibility (or bioavailability in case of *in vivo* models) measured with different models, the bioaccessibility of lead in soil samples was determined relative to the bioaccessibility of a soluble reference material. The reference substance used in this study was lead acetate, a readily soluble lead salt of which the bioaccessibility and bioavailability was studied both *in vitro* and *in vivo*, with the same method and at the same time as was done with the soil samples (see Figure 2). Using the relative bioaccessibility (or bioavailability) of lead from soil is the common way to compare *in vitro* with *in vivo* models (Denys et al., 2012; US EPA, 2007) and this was also done in the present study.

In addition, the oral bioavailability of lead acetate was determined from the ratio between the bioavailability of orally dosed and intravenously dosed lead acetate. Oral lead acetate was not dosed via drinking water, but via a small portion of feed, which could reduce the bioavailability. Currently, the bioavailability of lead acetate from drinking water is used as standard bioavailability in risk assessment.

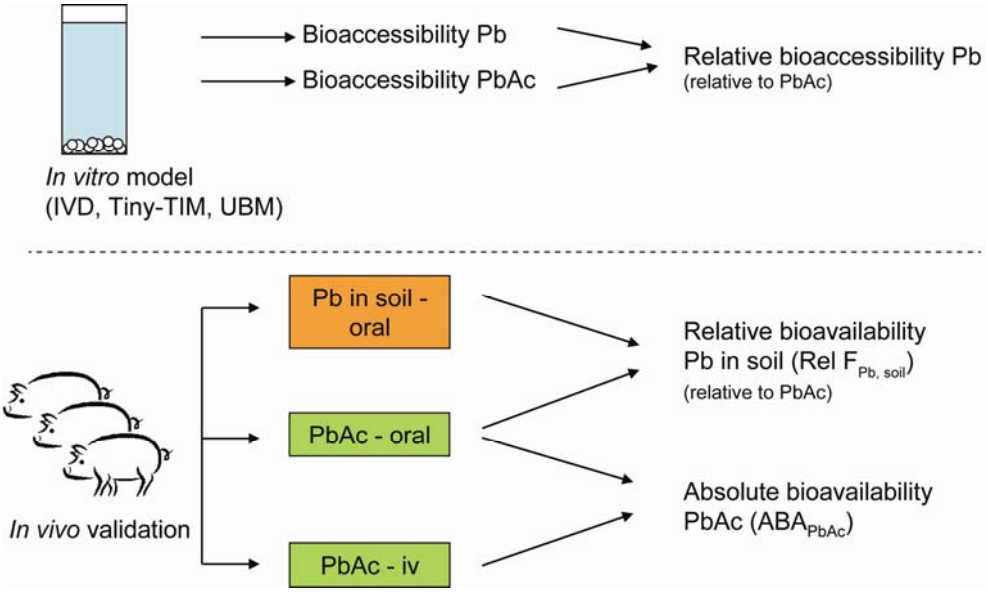

Figure 2 Overview of experimental studies and derivation of relative bioaccessibility and absolute and relative bioavailability. To compare the different models the relative bioavailability is used. For the determination of the absolute bioavailability lead acetate was also dosed intravenously. Pb: lead, PbAc: lead acetate, iv: intravenous, ABA: absolute bioavailability.

2 Soil samples

2.1 Characteristics soil samples

Soil with different characteristics and lead contamination was sampled from seven locations in the Netherlands (Table 1) based on a previous study by Hagens et al. (2009). All samples were dried (40°C) and sieved; only particles smaller than 2 mm were used. This fraction is based on Dutch legislation where maximum tolerable levels are expressed as concentrations of lead in the soil of < 2 mm (Dutch standard NEN5709:2006; Sample preparation for the determination of organic and inorganic parameters in soil). In many other countries (e.g. UK, France, USA) particle fractions of < 250 µm are generally used, as U.S. EPA considers particles < 250 µm to be the most likely to adhere to hands and be ingested by children (US EPA, 2000; US EPA, 2007).

Soil samples were characterized for dry matter, organic matter, clay and pH according to AS3010 (AlControl, the Netherlands). Calcite (carbonate content) was determined according to methods by AlControl (Rotterdam, the Netherlands). The characteristics are presented in Table 1. The lead source, the primary and secondary lead phases, and the chemical composition and size of the lead phases in the samples were previously determined by Hagens et al. (2009). These analyses were performed in other samples that originated from the same made ground sample locations. For this reason, they will be regarded as providing an indication for the current samples.

Table 1. Soil characteristics of the sampled Dutch made grounds.

Location	**Original soil type**	**Dry matter (DM)**	**Calcite**	**Organic matter**	**Clay**	**pH ($CaCl_2$)**
		% weight	% $CaCO_3$	% DM	% DM	
The Hague*	Dune sand	99.2	1.5	3.1	1.4	6.7
De Rijp	Marine sand / clay	93.1	0.6	7.4	11	6.7
Leiden	Fluviatile sand / clay	99.0	1.5	4	3.2	6.9
Maastricht	Loess	98.4	10.0	6.5	4.8	6.9
Nijmegen	Aeolian sands	99.2	13.0	2.8	1.8	7.1
Rotterdam	Marine sand / clay	97.2	1.7	9.5	13	6.8
Utrecht	Fluviatile sand / clay	98.8	2.6	4.8	5.5	6.5

* This soil was only used in the *in vitro* models, not in the *in vivo* study.

2.2 Lead concentrations soil

The amount of lead in the soil samples was determined using three methods:

- X-ray fluorescence (XRF) conducted by the British Geological Survey (BGS) according to BS EN ISO17025. Summarized, the samples were milled to produce a fine powder and further pressed into pellets. The concentration of Pb was determined by analysis of pressed powder pellets using a PANalytical Axios maX WD-XRFS fitted with automatic sample changer. The spectrometer was fitted with a 60 kV generator

and 4 kW rhodium end–window X-ray tube. The instrument was calibrated using a set of synthetic standards (Pro-Trace).

- Inductively coupled plasma mass spectrometry (ICP-MS) with *aqua regia* extraction. The total lead concentration was detected by a method based on the microwave-assisted destruction of the soil according to NEN 6961 using a 1:3 dilution of *aqua regia* with distilled water. Following the destruction, the soluble lead in the acidic mixture was detected with ICP-MS according to NEN-ISO 17294-2. The extraction and analysis were conducted at TNO Utrecht.
- ICP-MS with HNO_3 extraction. The total lead concentration was detected based on NEN 6961 using 0.43 M HNO_3 solution. Following the destruction, the soluble lead in the acidic mixture was detected with ICP-MS according to NEN-ISO 17294-2. The extraction and analysis were conducted at TNO Utrecht.

In the Netherlands, the total lead concentration in soil is commonly determined by extraction with *aqua regia*. The milder extraction with 0.43 M HNO_3 was performed to investigate whether this method could predict the bioaccessibility of the metals in the soil. Validation of the *aqua regia* extraction was performed using X-ray fluorescence (XRF) analysis. *Aqua regia* cannot liberate the lead that is very tightly bound to soil particles, and the efficacy of the extraction is dependent on the duration and temperature used, whereas XRF detects all lead present in a soil sample.

The results of each method are presented in Table 2, and show that for most samples the lowest lead concentration was, as expected, for the method using 0.43 M HNO_3 and the highest for XRF. Note that the results for these methods were obtained from different subsamples from a sampled soil and that there might have been differences due to inhomogeneity of the soils. This appears to be the case for the sample locations Nijmegen and Utrecht. Lead concentrations from these two locations are highly variable and in addition, they are higher in the HNO_3 extracts than in the *aqua regia* extracts, which cannot occur when testing the same, homogeneous subsample.

Table 2. Lead concentrations (mg/kg) ± SD in the test soils, based on three methods (n = 3)

Location	**Lead concentration (mg/kg)**		
	Aqua regia	0.43 M HNO_3	XRF
The Hague[*]	604, 673[a]	526 ± 58	662 ± 112
De Rijp	1138 ± 104	975 ± 55	1370 ± 232
Leiden	522 ± 28	468 ± 33	706 ± 119
Maastricht	1021 ± 169	593 ± 29	991 ± 167
Nijmegen	2572 ± 76	3014 ± 578	4285 ± 724
Rotterdam	2111 ± 68	1887 ± 78	2317 ± 392
Utrecht	2842 ± 399	3573 ± 865	3567 ± 603

[*] This soil was only used in the *in vitro* models, not in the *in vivo* study

[a] n=2

3 Pilot study - materials and methods

3.1 In vitro digestion (IVD) model

3.1.1 First pilot experiment

The main aim of the first pilot experiment was to investigate the reproducibility of the bioaccessibility using different amounts of soil in the IVD model. A second aim was to identify the influence of the separation method on the bioaccessibility of lead by testing two separation techniques, i.e. centrifugation and ultrafiltration.

The IVD model is a static gastrointestinal model. The model was developed by RIVM based on the model of Rotard et al. (1995) and is currently operated by RIKILT (Wageningen UR). The model simulates the bioaccessibility of a substance (F_B), which is the amount of a substance that can be maximally absorbed. Digestive juices are prepared artificially and the composition is based on human physiology. The digestive juices are added to a soil sample according to physiological transit times and are mixed thoroughly. The rationale for choosing the number of simulated compartments of the gastrointestinal tract, temperature, soil-to-fluid ratio, ratio of digestive juices, transit times, centrifugation, pH values, mixing, constituents and their concentrations, and bile, are addressed in Oomen et al. (2003). The initially developed *in vitro* digestion model simulates fasted conditions of the human gastrointestinal tract; in follow-up experiments the model was developed for simulation of fed conditions of the human gastrointestinal tract (Versantvoort, 2004; Versantvoort et al., 2005). Since children are mostly in a semi-fed state, in the present study a semi-fed model was used (Figure 3), which has a pH and concentrations of constituents with values in between the fasted and the fed model.

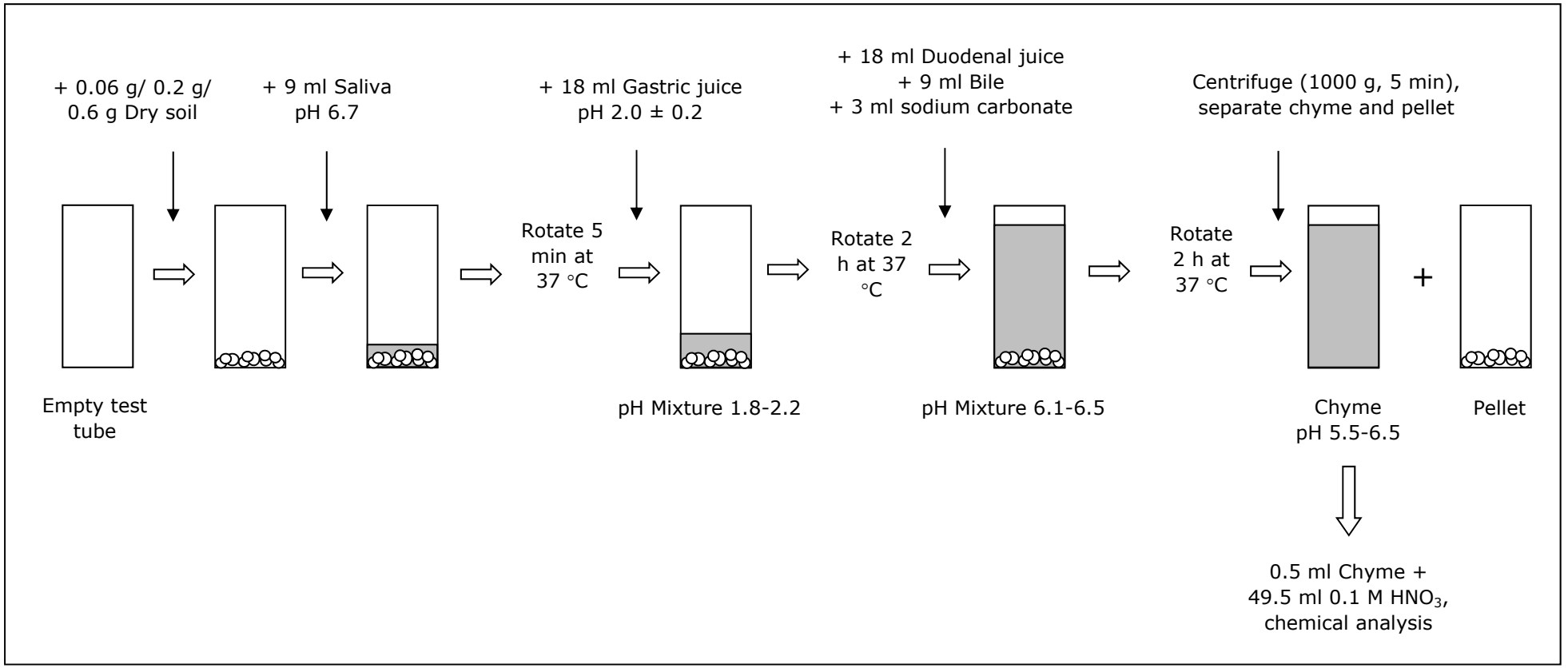

Figure 3 Schematic representation of the in vitro digestion (IVD) model simulating semi-fasted conditions

Different amounts of soil were tested with the semi-fed model (for details see Appendix 1) with or without adding food and using different solid-to-fluid ratios:

- 0.06 g soil, standard model (ratio 1:1000)
- 0.6 g soil, standard model (ratio 1:100)
- 0.2 g soil, standard model (ratio 1:300)
- 0.2 g soil, up-scaled model (ratio 1:1000)

Although the use of 0.06 g soil in the standard model has the preferred solid-to-fluid ratio, it is a very small amount and it may be questioned if this is sufficient for obtaining a reproducible bioaccessibility value, due to inhomogeneity of the soil sample. For this reason, various amounts of soil and upscaling of the used volumes were investigated to determine the optimal conditions for further testing. In addition, the effect of the separation technique on the bioaccessibility of lead was investigated. In addition to centrifugation as a method to separate the chyme from the solid residues, ultrafiltration (10 kDa filter column) was also tested.
As the soils for the current validation study in swine had not yet been sampled at the start of the first IVD pilot experiment, samples from the previous study from the location Leiden (#29) were used (Hagens et al., 2009).

3.1.2 Second IVD pilot experiment

In a second IVD pilot experiment, it was tested whether:
1) separation of the chyme by microfiltration is suitable for determination of the bioaccessibility,
2) the solid-to-fluid ratio at 0.2 g should be 1:300 or 1:1000, to further determine the influence of the volumes on the bioaccessibility and,
3) which amount of lead acetate is most suitable for further use as a reference compound in the *in vivo* study.

Three experimental conditions were tested:

- Standard model (soil-to-fluid ratio 1:300), separation by centrifugation
- Standard model (soil-to-fluid ratio 1:300), separation by microfiltration
- Upscaled model (soil-to-fluid ratio 1:1000), separation by centrifugation

The standard and upscaled model (both with 0.2 g) were carried out as described in Appendix 1. The chyme was separated from the fraction that is too large to be absorbed in the intestine either by centrifugation for 5 min at 2900 g or by filtration using a 0.45 µm cellulose acetate disk filter. The chyme samples were diluted with 0.1 M HNO_3 in a ratio of 1:100 and stored at ≤-20°C until analysis with ICP-MS.

3.2 *In vitro* Tiny-TIM system

TIM (TNO gastro-Intestinal Model) is a dynamic *in vitro* model simulating the gradual transit of a meal through the gastrointestinal tract including emptying curves, pH profiles and secretion of the different digestion fluids (Minekus et al., 1995) (Figure 4). A simplified TIM system called Tiny-TIM was previously validated for the determination of protein digestion and bioaccessibility of amino acids (protein quality) in foods and digestion of carbohydrates (Schaafsma, 2005; Havenaar et al., 2013) and also used to determine bioaccessibility of lead from soil (Hagens et al., 2009). The Tiny-TIM model was also used in the current study, however, no food was added during any of the experiments.

The Tiny-TIM system was used as described by Hagens et al. (2009), with some modifications. Duplicate experiments were performed in the Tiny-TIM system, with simulation of the gastrointestinal conditions of young children between two meals. The different soils were introduced into the gastric compartment together with water and artificial saliva (total intake of 125 g). The simulated gastrointestinal parameters were, among others: body temperature of 37°C; mixing of the gastric and intestinal contents by peristaltic movements; the pH curve in the stomach compartment in relation to the secretion of gastric acid (from pH 2.8 to 1.7 in 120 min); the kinetics of gastric emptying (halftime was 60 minutes). The gastrointestinal transit time was 6 h. After 4 h a glass of water was added to the stomach, simulating the intake of drinking water and supporting the emptying of the soil from the stomach into the small-intestinal compartment. A semi-permeable membrane unit (cut-off of 5-7 kDa) was connected to the intestinal compartment (Figure 4) for the continuous dialysis of digested, released and dissolved small MW compounds (e.g. Pb) and water into the dialysis liquid[2].

3.2.1 Tiny-TIM pilot experiment

In the pilot study, three soils were tested from the locations The Hague Rotterdam, and Nijmegen (n=2) and a negative control (water) in portions of 0.5 and 5 g.
The dialysis liquid was collected in a HNO_3 solution (final concentration 0.1 M). Pooled samples (0-4 h and 0-6h) were frozen in duplicate at ≤ -18°C. The dialysis liquid samples were analyzed for the concentration of lead to calculate the bioaccessible amount of lead. At the end of the experiments, for all soil samples the total residues were sampled, mixed with HNO_3 (final concentration 0.1 M) and stored at ≤ -18°C. The residues were analyzed for total lead (n=1) to determine the recovery of the Pb from the Tiny-TIM system.
Before addition of HNO_3 to the residue, first a 50 ml fraction of the intestinal residue was centrifuged (2900 g, 5 min) and 2.5 ml of this supernatant was diluted in 2.5 ml 0.2 M HNO_3 and stored at ≤ -18°C for analysis. The remainder was added to the residue again.

[2] Note that the TIM systems mimic the bioaccessibility x passive absorption (F_B x F_A) of a substance, in contrast to the static models used in this study, which simulate bioaccessibility (F_B) only. The TIM model does not mimic active transport of compounds across the intestinal tract.

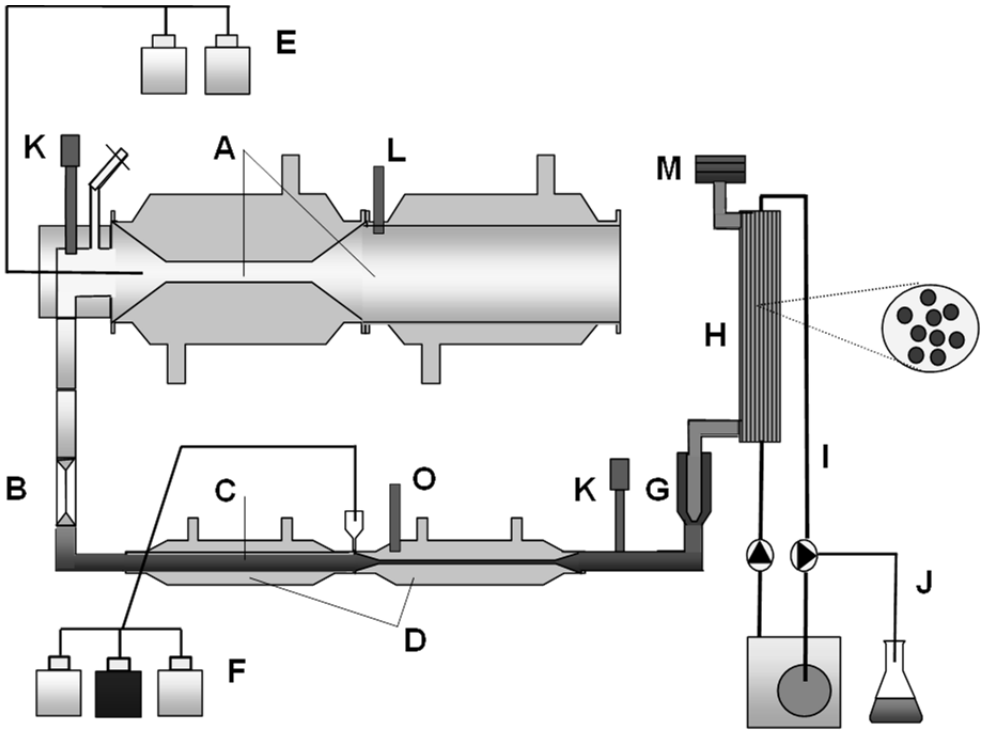

Figure 4 Schematic view of the Tiny-TIM system: A, gastric compartment; B, pyloric sphincter; C, chyme; D, small-intestinal compartment; E, gastric secretion; F, intestinal secretion; G, pre-filter; H, semi-permeable membrane; I, dialysis liquid; J, water absorption; K; pH electrodes; L, pressure sensor; M, level sensor; O, temperature sensor.

3.3 *In vivo* pilot experiment

Six 5-week-old juvenile male pigs (obtained from Verbeek, Lelystad) were housed in pairs and acclimatized for one week prior to the experiment. Pigs received two meals per day (slurry with feed: water ratio of 1:2.5) from one batch in the morning (0800h) and in the afternoon (1600h). No drinking water was available between the morning and afternoon meal. Pigs had free access to tap water in the period between the afternoon and morning meal. Clinical symptoms were scored daily and body weights were measured every other day. The study was agreed upon by the institute's ethical committee on experimental animals, in accordance to national legislation.

Animals were dosed with 0, 25, 50, 150, 300, 750 µg/kg bow/day lead acetate solution mixed in feed (standard pig feed, Abdiets) with one animal per dose group. Lead acetate was administered at 13.00h on day 1 until day 7, followed by an elimination period from day 8 until day 14. During dieting and feeding of lead acetate, the pigs were separated to prevent cross-contamination. Blood samples were collected daily from the jugular vein at 11.00h on day 1 until 14. Samples were collected in heparin-coated Vacutainer tubes and stored on ice for the maximal duration of one hour. Thereafter, blood samples were stored at -20°C until analysis. At Day 15, animals were anesthetized and subsequently euthanized by exsanguination. Macroscopic observation of the intestine was performed on all animals, and samples of the liver, kidney and femur were

collected and stored at -20°C until extraction and analysis for lead concentration.

3.4 Extraction and analysis of lead in chyme, dialysate, blood and tissue

The extraction of lead from blood, soft tissues, and femur was based on methods as described by Casteel et al. (2006), Koyashiki et al. (2010), Smith et al. (2009), Martena et al. (2010) and Perello et al. (2008); see Appendix 2 for details.
The soluble lead in the extractions from blood, liver and kidney (*in vivo* experiment) and the chyme, dialysate and small-intestinal residue samples (*in vitro* experiments) was detected with ICP-MS according NEN-ISO 17294-2.

4 Pilot study - results

4.1 IVD model

4.1.1 First pilot experiment

Reproducibility

The bioaccessibility values for the different amount of soil in the different models and corresponding relative standard deviation (RSD) are presented in Table 3. The reproducibility of the eight 0.06 g soil samples was low, shown by the relatively high RSD. Increasing the amount of soil to 0.2 or 0.6 g soil (n=8) resulted in an increased reproducibility in the models without food, with the lowest variation observed with 0.2 g soil in the upscaled model (n=4). The effect of food addition on the bioaccessibility and reproducibility was inconclusive.

Table 3. Reproducibility of different amounts of soil in the semi-fed IVD model.

	Bioaccessibility (%) without food		**Bioaccessibility (%) with food**	
	Mean	**RSD**	**Mean**	**RSD**
0.06 g soil, standard model, n=8				
Location De Rijp (#17) [a]	20	5.5	26	22
Location Leiden (#29) [a]	39	13	46	15
Location Montana (2710a)[b]	18	21	32	11
0.6 g soil, standard model, n=8				
Location Leiden (#29)[a]	31	9.4	21	6.5
0.2 g soil, standard model, n=8				
Location Leiden (#29)[a]	45	8.5	32	12
0.2 g soil, upscaled model, n=4				
Location Leiden (#29)[a]	42	4.9	50	15
Lead acetate, n=2				
0.05 mg	53, 58		71, 75	
5 mg	73, 52		68, 77	

[a] Soil sample from previous study (Hagens et al., 2009)
[b] Reference soil

The bioaccessibility measured for samples from De Rijp and Leiden was comparable with the results from a previous study (Hagens et al., 2009), in which a bioaccessibility of 26% (RSD 0.3%) for location De Rijp (#17) and 42% (RSD 7.0%) for location Leiden (#29) was measured using 0.06 g soil in the fasted IVD model. The bioaccessibility measured with 0.6 g soil (with and without food) and 0.2 g soil (standard model, with food) was lower as compared with 0.06 g soil. All other test conditions resulted in comparable bioaccessibilities. The results of the first pilot experiment show that the amount of soil clearly influences the bioaccessibility and its reproducibility and further experiments are conducted with 0.2 g soil, standard and upscaled model.

Separation techniques
The influence of the separation technique on the reproducibility was investigated for centrifugation and ultrafiltration, using 0.2 g soil from location Leiden (standard model). Centrifugation resulted in a bioaccessibility of 45% without food and 32% with food (Table 3). The bioaccessibility after ultrafiltration was very low with only 1% bioaccessibility in conditions with and without food. Therefore, ultrafiltration was excluded from further experiments.

Bioaccessibility of lead acetate
The bioaccessibility of lead was determined by addition of 0.05 or 5 mg lead acetate, with and without food, to the semi-fed IVD model. The bioaccessibility of 0.05 and 5 mg lead acetate in a model without food was 55% and 62% respectively and in a model with food 73% for both amounts of lead acetate. These results are quite comparable to the bioaccessibilities found previously by Hagens et al. (2009), where samples with spiked lead acetate gave a bioaccessibility of 51-53% under fasted conditions and 40-58% under fed conditions.

4.1.2 Second pilot experiment

Table 4 shows that the bioaccessibility of PbAc in the standard semi-fed IVD model with separation by centrifugation is between 8% and 30%, which is considerably lower than the historical range (52% for fasted and 40-57% for fed model, Hagens 2009) and lower than the values found in the first pilot experiment. In addition, the relative standard deviation is large (RSD of 55%). Also, results for the Montana 2710A soil were somewhat lower than before.
A possible cause for the high variation in the lead acetate samples is the formation of precipitation containing lead, caused by freezing the samples, which was visually observed after thawing the samples. In future studies, samples will be stored at room temperature and analyzed for lead concentration within 1-2 days, which will prevent the precipitation of lead salts.
Due to the low values and the large variation, these results for PbAc were considered unreliable and were not used for further calculations of the bioaccessibility relative to PbAc.

The results of the first pilot experiment suggest that 0.2 g soil does not affect the bioaccessibility and is a suitable amount to put into the model. Nevertheless, this was only based on one soil type (Leiden sample from Hagens et al. (2009)). For this reason, in the second pilot experiment, three other soils (De Rijp, Utrecht and Leiden, from Hagens et al. (2009)) were tested in a standard and upscaled model with 0.2 g soil. These soils were selected based on their different expected bioaccessibilities (low for De Rijp, high for Utrecht and intermediate for Leiden). Although the upscaled model resulted in slightly higher bioaccessibilities than the standard model, the upscaling of digestion fluid volumes does not seem to be essential for determining a reliable bioaccessibility. For the validation study, it was decided to use the standard semi-fed model.

Separation by microfiltration resulted in very low bioaccessibilities ranging from 1.1 to 4.2%. These values are too low to give good detectable results. Moreover, such low bioaccessilibities for lead from soil are considered unlikely based on knowledge of the bioavailability of lead in humans (Maddaloni et al., 1998). This may be explained by the active transport of lead in the gastrointestinal tract of humans. Microfiltration simulates only passive transport and underestimates the

actual absorbed fraction, while centrifugation also includes lead (reversibly) bound to large complexes which can potentially be taken up. For these reasons, the use of microfiltration to separate the chyme was excluded from the follow-up study.

Table 4. Bioaccessibility of lead from soil (± RSD) determined in chyme samples obtained from the IVD model (in triplicate).

			Bioaccessibility[a] (%)		
	Sample	**Amount**	**Standard model[b] - centrifugation**	**Upscaled model[c] - centrifugation**	**Standard model[b] - microfiltration**
1	PbAc	0.05 mg	24 ± 26	33 ± 31	
2	PbAc	0.05 mg	22 ± 5	25 ± 24	
3	PbAc	0.05 mg	20 ± 16	40 ± 15	
1	PbAc	0.5 mg	10 ± 55	17 ± 22	
2	PbAc	0.5 mg	11 ± 41	16 ± 5	
3	PbAc	0.5 mg	8.2 ± 22	16 ± 18	
1	PbAc	5 mg	30 ± 10	44, 51 [d]	
2	PbAc	5 mg	26 ± 7	34, 34 [d]	
3	PbAc	5 mg	29 ± 18	40, 42 [d]	
1	Montana	0.2 g	13 ± 29	-	1.8 ± 53.3
2	Montana	0.2 g	13 ± 23	-	1.4 ± 43.4
3	Montana	0.2 g	14 ± 18	-	1.1 ± 45.1
1	Utrecht	0.2 g	20 ± 5	25 ± 14	2.6 ± 24.1
2	Utrecht	0.2 g	22 ± 27	32 ± 4	4.2 ± 35.8
3	Utrecht	0.2 g	20 ± 13	32 ± 34	2.6 ± 19.8
1	De Rijp	0.2 g	15 ± 18	25 ± 11	2.8 ± 19.9
2	De Rijp	0.2 g	12 ± 17	23 ± 20	3.7 ± 56.9
3	De Rijp	0.2 g	22 ± 23	24 ± 8	2.6 ± 19.8
1	Leiden	0.2 g	25 ± 9	27 ± 17	4.1 ± 43.1
2	Leiden	0.2 g	17 ± 35	22 ± 7	2.5 ± 32.7
3	Leiden	0.2 g	19 ± 4	12 ± 22	3.2 ± 31.2

[a] Values represent the average of triplicates ± RSD.
[b] Solid to fluid ratio is 1:300
[c] Solid to fluid ratio is 1:1000
[d] n=2

4.2 Tiny-TIM model

A pilot study for the Tiny-TIM system was performed to examine the possibility of using a shorter running time (4 h) than usual (6 h), and to determine the optimal amount of soil to be tested. Residues (remainder in model after 6h) were sampled to be able to determine the lead recovery from the system (performed for a limited number of runs). Furthermore, a fraction of the intestinal residue was centrifuged according to RIVM settings to enable a comparison between the IVD model and the Tiny-TIM system.

A difference between the lead concentrations measured in the 0-4 h samples and 0-6 h samples was observed. The 0-6 h samples showed a higher bioaccessibility compared to the 0-4 h samples (data not shown). Therefore, it was decided to use a 0-6 h running time for the pilot experiment and the validation experiments.

The amount of soil added to the Tiny-TIM system influenced the lead bioaccessibility. The experiments in which 0.5 g soil was added resulted in a 4-7 fold higher bioaccessibility (BAc) as compared to addition of 5 g soil (Table 5). To mimic a worst-case and more realistic intake of the amount of soil, it was decided to test 0.5 g soil in the validation study.
To determine the lead recovery (Table 5) from the system, the total amount of lead in the gastric plus intestinal residues and dialysate was determined, summed and compared to the total amount that was present in the amount of soil brought into the gastric compartment of the system (n=1; lead intake was based on the extractions with *aqua regia*).

Table 5. Bioaccessibility (BAc) of lead from soil determined in dialysate (n=2) in the pilot experiment using the Tiny-TIM system.

Sample	Amount soil (g)	Lead intake (µg)	BAc dialysate (%)	Recovery of Pb in total residue (%)[a]
The Hague	5	3190	3.8, 3.4	52.5
The Hague	0.5	319	12.9, 17.2	55.4
Rotterdam	5	10554	2.6, 2.2	47.5
Rotterdam	0.5	1055	16.8, 15.6	74.5
Nijmegen	5	12860	3.7, 3.4	125.2
Nijmegen	0.5	1286	14.0, 25.1	291.8

[a] Recovery of lead from soil based on total residues + dialysate was based on a single experiment (second run)

The high recovery found for the Nijmegen runs is most likely due to non-homogenous distribution of lead over the soil sample. This was only tested as n=1. Because recovery was not measured in the other methods, it was decided to take no further actions and to not exclude this result.
The recovery for The Hague and Rotterdam (n=1) is below 90-100%, indicating that either not all soil can be sampled out of the system, or not all lead can be measured (despite the used destruction method for the residue samples). It indicates that thorough cleaning of the model after each run is necessary.

4.3 *In vivo* study

No clinical signs were observed in any of the dose groups. Body weight gain was comparable between the different dose groups. Figure 5 shows the lead concentrations in blood over time as determined with ICP-MS. At a dose level of 150 µg/kg bw/day, the measured concentrations clearly exceeded the control levels. During the exposure phase of the upper three dose levels, lead concentration in blood gradually increased. Blood lead concentration decreased during the elimination phase.

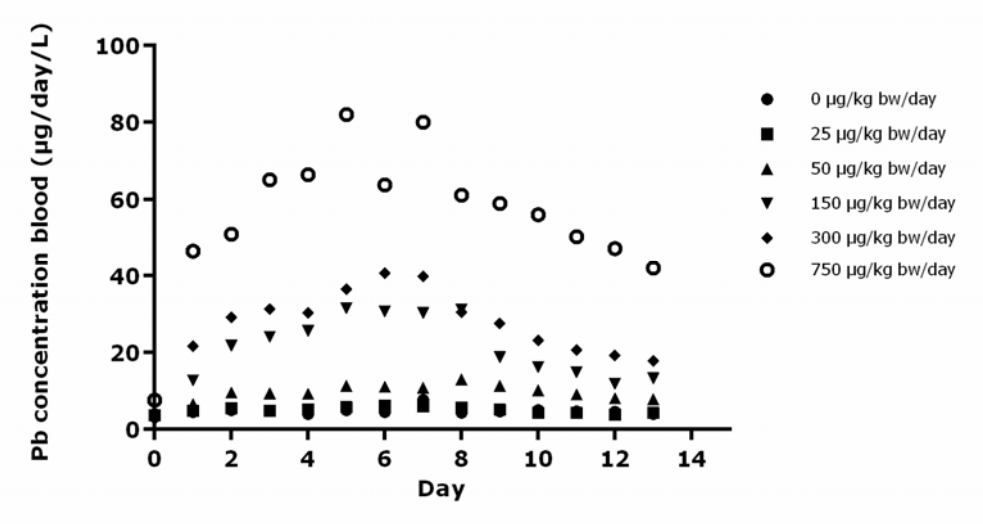

Figure 5 Lead concentrations in blood of pigs (n=1 per dose) orally treated with lead acetate.

Clear dose response patterns were observed for blood, femur, liver and kidney (Figure 6). Lead concentrations in pig feed were below the limit of detection.

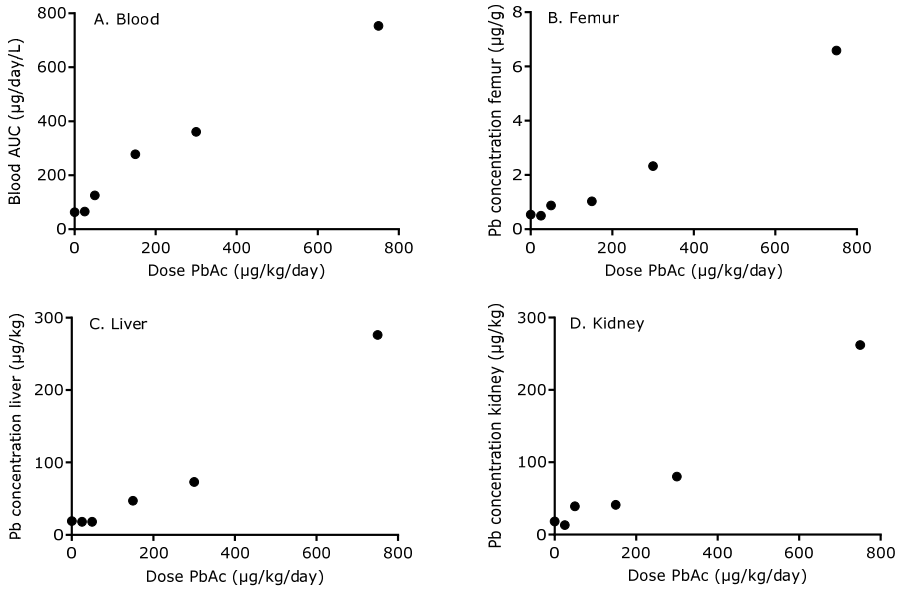

Figure 6 Dose response curves of lead in blood (A), femur (B), liver (C) and kidney (D) from swine (n=1 per dose) administered with lead acetate.

The validation study will be performed with 150 µg/kg bw/day lead acetate as a reference. This concentration results in a detectable level of lead in the blood and tissues, above the control level, and it is sufficiently low to prevent saturation by binding to blood (Casteel et al., 2006). Lead concentrations will be determined in blood and liver.

5 Validation study – materials and methods

5.1 In vitro digestion (IVD) model

For the validation study, the standard model (soil-to-fluid ratio 1:300) with separation by centrifugation was used, based on the results of the pilot study. The seven soil samples and the Montana 2710A soil (n=4) were studied. PbAc (n=4) was included in two doses: 0.1 and 0.5 mg. The study was performed with 0.2 g soil under semi-fed conditions (without adding food) as described in section 3.1. The samples were collected in 0.1 M HNO_3 in a 1:100 dilution, except for the blank samples and PbAc 0.1 g samples that were diluted at a 1:10 ratio. The chyme samples were stored at room temperature and were analysed with ICP-MS within one day after isolation.

5.2 Tiny-TIM model

The Tiny-TIM system was used as described above in sections 3.2 and 4.2. Soil samples from Leiden, Maastricht, De Rijp and Utrecht (0.5 g) were used, tested in duplicate (the other soils had been tested in the pilot experiment). The dialysate was collected after a 6 h digestion time. PbAc (1 ml containing 1 mg/ml Pb; pH 4.5) was included as a reference.

In addition, from all soil runs and for the PbAc reference the total residues were sampled, mixed with HNO_3 (final concentration 0.1 M) and stored at ≤-18°C. Only the residues (n=2) of the PbAc runs were analyzed for total lead to determine the recovery of Pb from the Tiny-TIM system. Furthermore, to compare the bioaccessibility measured in Tiny-TIM and IVD a fraction of the intestinal residue of the Tiny-TIM model was centrifuged in the same manner as the IVD samples. The Pb in the supernatant was measured and added to the Pb amount present in the dialysate of Tiny-TIM, to obtain a value 'corresponding' to the bioaccessibility value of the IVD model.

5.3 Unified BARGE Method

The Unified BARGE Method (UBM) is based on the IVD model, with a few adaptations. See for the compositions of digestive solutions Wragg et al. (2011) and for a detailed description of the method the supplementary information in Denys et al. (2012). In short: The temperature was maintained at 37°C throughout the procedure. Nine mL of saliva was added to 0.6 g of soil and the suspension was shaken by hand for 10 seconds. Then, 13.5 mL of gastric solution was added to the soil suspension and the pH of the solution was measured and adjusted to 1.20 ± 0.05 by the addition of HCl (37%) or NaOH (1.0M). After an initial 1 h incubation time, samples were taken and analyzed to obtain the bioaccessibility of lead in the gastric phase. To simulate the intestinal phase, 9 mL of bile and 27 mL of duodenal fluids were added and the pH was adjusted to 6.3 ± 0.5. The suspension was mixed end-over-end for a further 4 hours at 37ºC. The gastrointestinal phase was then removed for analysis by careful pipetting after centrifuging the suspension at 4500 g for 15 minutes and acidification by the addition of 1.0 mL HNO_3 (67%). The experiments were carried out in triplicate.

5.4 *In vivo* study

Juvenile male pigs (6 per dose group) were dosed with 0.4 or 1 g soil per kg body weight to achieve a blood concentration that corresponds to the intake of

approximately 150 µg lead/kg bw/day. Soil samples from Utrecht were divided into two dose levels with three animals per dose group (Table 7). Animals were dosed at day 1 until day 7 by mixing the soil with feed, followed by an elimination period from day 8 until day 14. Blood samples were collected from the jugular vein at day 1, 2, 4, 5, 7, 8, 9, 11, 12 and 14. Samples were collected two hours before dosing in heparin-coated Vacutainer tubes and stored on ice for the maximal duration of one hour. Thereafter, blood samples were stored at -20°C until analysis. At day 15, all animals were anesthetized and subsequently euthanized by exsanguination. Macroscopic observation was performed on all animals and samples of the liver were collected and stored at -20°C until analysis.

Table 7. Treatment groups for in vivo validation study.

Group	# pigs	Treatment	Amount soil	Dose lead[a]	Route
			g soil / kg bw	µg/kg bw/day	
1	4	Negative control	-	-	Oral
2	6	PbAc	-	150	IV
3	6	PbAc	-	150	Oral
4	6	Rotterdam	0.4	844	Oral
5	6	De Rijp	1	1138	Oral
6	6	Leiden	1	522	Oral
7	6	Maastricht	1	1021	Oral
8	6	Nijmegen	0.4	1029	Oral
9A	3	Utrecht	0.4	1137	Oral
9B	3	Utrecht	1	2842	Oral
Total	52				

[a] Calculated based on the total lead concentration in soil as determined by extraction with *aqua regia*.

5.5 Relative bioaccessibility

For comparison of the bioaccessibilities measured in the different models, the results are expressed as the relative bioaccessibility or relative bioavailability, i.e. relative to that of the reference compound lead acetate.

The relative bioaccessibility (RBAc, *in vitro*) for the IVD model (chyme), Tiny-TIM model (dialysate) and UBM (chyme) was calculated by the ratio of the bioaccessibility of lead from soil and the bioaccessibility of PbAc, corrected for the lead dose, according to equation 2 and 3:

$$\text{RBAc} = \frac{\text{Pb in chyme} / \text{Dose}_{\text{Pb}}}{\text{PbAc in chyme} / \text{Dose}_{\text{PbAc}}} \times 100 \qquad [2]$$

$$\text{RBAc} = \frac{\text{Pb in dialysate} / \text{Dose}_{\text{Pb}}}{\text{PbAc in dialysate} / \text{Dose}_{\text{PbAc}}} \times 100 \qquad [3]$$

The relative bioavailability (RBA, *in vivo*) of lead from soil determined in blood and liver are calculated according to equation 4 and 5 respectively:

$$\text{RBA} = \frac{\text{AUC}_{\text{Pb, oral}} / \text{Dose}_{\text{Pb}}}{\text{AUC}_{\text{PbAc, oral}} / \text{Dose}_{\text{PbAc}}} \qquad [4]$$

$$\text{RBA} = \frac{\text{Liver Pb}_{\text{oral}} / \text{Dose}_{\text{Pb}}}{\text{Liver PbAc}_{\text{oral}} / \text{Dose}_{\text{PbAc}}} \qquad [5]$$

Where:

AUC = area under de curve

6 Validation study – results

The bioaccessibilities and bioavailabilities in the validation study are calculated based on the lead concentrations in soil as determined by *aqua regia* extraction, as this is the method used in location-specific soil investigations in the Netherlands. Calculations based on XRF analyses are included in Appendix 3. The lead concentrations in soil from The Hague, De Rijp, Maastricht, and Rotterdam, as determined by XRF, are comparable to those determined by *aqua regia* extraction and lead to similar bioaccessibilities and bioavailabilities. However, lead concentrations in soil from Leiden, Nijmegen, and Utrecht, as determined by XRF, were 1.3-1.7x higher than for *aqua regia* extraction, leading to lower bioaccessibilities and bioavailabilities and subsequently to differences in correlation between *in vitro* and *in vivo* data.

6.1 IVD model

The absolute bioaccessibilities (BAc) for the seven Dutch made grounds measured in the IVD model varied between 18% and 60% (n=4), (Table 8). Remarkably, both PbAc concentrations resulted in very low bioaccessibilities of 0.5% and 5%. The cause of these low values is not clear. Hence, these data were not considered suitable for further calculation of the RBAc. For this reason, the PbAc results from the first IVD pilot study were used to determine the RBAc (Table 8). The RBAc in the seven soils varied between 30% and 102%.

Table 8. Absolute (BAc) and relative (RBAc) bioaccessibility (± RSD) of made ground samples determined by IVD model.

Sample	Amount soil (g)	Lead intake (µg)[a]	BAc chyme (%)	RBAc chyme (%)[b]
Montana	0.2 g	1085	24	41 ± 1
The Hague	0.2 g	128	45	77 ± 42
De Rijp	0.2 g	228	60	102 ± 7
Leiden	0.2 g	104	53	90 ± 5
Maastricht	0.2 g	204	18	30 ± 25
Nijmegen	0.2 g	517	33	56 ± 45
Rotterdam	0.2 g	423	40	68 ± 9
Utrecht	0.2 g	568	60	102 ± 6
PbAc	0.1 mg	55	0.5	
PbAc	0.5 mg	273	5	
PbAc pilot[c]	0.05 / 5 mg		59 ± 16	-

[a] Based on lead concentration in soil as determined by *aqua regia* extraction.

[b] Bioaccessibility of lead from soil relative to bioaccessibility from PbAc as determined in the pilot study.

[c] PbAc value obtained from the first pilot IVD study.

6.2 Tiny-TIM model

Soil bioaccessibility values measured using the Tiny-TIM system were calculated based on the lead concentration in the dialysate, relative to the bioaccessibility measured for PbAc. The RBAcs (n=2) were, on average, 4 fold lower than obtained from the IVD model and ranged from 7% to 43%. Table 9 presents the

absolute and relative bioaccessibilities measured for the made grounds both in the pilot and validation study.

Table 9. Absolute and relative bioaccessibility of lead from soil determined by the Tiny-TIM model.

Sample	Amount soil (g)	Lead intake (µg)[a]	BAc dialysate (%)	RBAc dialysate (%)[b]
The Hague	0.5	319	12.9, 17.2	**28, 37**
De Rijp	0.5	569	9.8, 8.8	**21, 19**
Leiden	0.5	261	6.7, 5.6	**14, 12**
Maastricht	0.5	510	3.0, 3.5	**6, 8**
Nijmegen	0.5	1286	14.0, 25.1	**30, 54**
Rotterdam	0.5	1055	15.6, 16.8	**36, 33**
Utrecht	0.5	1421	21.0, 19.3	**45, 41**
PbAc	0.001	1000	46.9, 46.3	**-**

[a] Based on lead concentration in soil as determined by *aqua regia* extraction.

[b] Average bioaccessibility relative to PbAc

The total recovery of lead from the Tiny-TIM system as measured for PbAc (n=2) was 92.5%.

6.2.1 *Analyses of centrifuged residues for comparison with IVD*

To be able to compare the bioaccessibility measured in the IVD model and Tiny-TIM model, a fraction of the intestinal residue from Tiny-TIM was centrifuged in the same manner as the IVD samples. The amount of Pb in the dialysis liquid and in the intestinal residue were summed to obtain an 'IVD-like' BAc and RBAc.

The results show that the bioaccessibility from the dialysate and the centrifuged intestinal residue combined are closer to the findings from the IVD model than the dialysate only. The absolute and relative bioaccessibility obtained from the dialysate was on average a factor 4-5 lower than the bioaccessibility from the IVD model, however, combined with the centrifuged residue samples, the difference is reduced to an average factor of 1.5-1.8. These data indicate that the (remaining) difference between the Tiny-TIM system and IVD model may be largely explained by the difference between the separation methods used (semi-permeable membrane vs centrifugation).

Table 10. Absolute (BAc) and relative (RBAc) bioaccessibility of lead from soil determined in the centrifuged residue samples of the Tiny-TIM system.

Sample	BAc centrifuged residues (%)	BAc dialysate (%)	BAc total (%)	RBAc total (%)	BAc IVD (%)	RBAc IVD (%)
The Hague	21	15	36	50	45	77
De Rijp	15	10	25	35	60	102
Leiden	20	7	27	37	53	90
Maastricht	11	3	14	20	18	30
Nijmegen	ND	ND	ND	ND	ND	ND
Rotterdam	21	17	37	53	40	68
Utrecht	35	20	55	78	60	102
PbAc	24	47	71	-	59	-

6.3 Unified BARGE method

Bioaccessibilities of the Dutch made grounds using the UBM are presented in Table 11. The RBAc values of the gastric phase are higher than those of the gastric + intestinal phase, which is explained by the differing pH values of these two phases.
The RBAc for the gastric phase is in some cases > 100% (Leiden, Nijmegen and Utrecht). However, this is not the case when the soil concentrations are based on XRF data (see Appendix 3).

Table 11. Absolute (BAc) and relative (RBAc) bioaccessibility of lead from soil determined by UBM (n=3).

Sample	Lead intake (µg)[a]	BAc (%)	RBAc[b] (%) ± RSD	BAc (%)	RBAc[b] (%) ± RSD
		Gastric		Gastric + intestinal	
The Hague	638	83	84 ± 4	28	43 ± 19
De Rijp	1138	94	95 ± 4	25	38 ± 8
Leiden	522	119	120 ± 5	39	59 ± 18
Maastricht	1021	17	17 ± 7	6	10 ± 29
Nijmegen	2572	153	154 ± 1	48	73 ± 55
Rotterdam	2111	86	87 ± 3	21	31 ± 3
Utrecht	2842	108	110 ± 7	49	74 ± 7
PbAc [c]		99		66	

[a] Based on *aqua regia*

[b] Average bioaccessibility relative to PbAc

[c] Bioaccessibility of PbAc was obtained from previous experiments (Denys et al., 2012)

6.4 *In vivo* validation

The bioavailability of lead in swine was calculated based on the lead concentrations in blood (AUCs) and livers from swine that ingested soil samples or PbAc for seven days.
The AUCs of the group of swine that were orally dosed with the reference material PbAc (150 mg/kg/d) were a factor 2 lower than in the pilot study. Compared with the intravenously dosed group of swine (representing 100% bioavailability of lead acetate), the bioavailability of the orally dosed lead acetate appeared to be only 7% (pilot study: 13%). Furthermore, the concentrations in livers of this group of swine were so low that they barely exceeded the concentrations in the control group. For this reason, and because the liver data correlated well with the blood data (r^2 = 0.94) it was decided not to calculate relative bioavailabilities (RBAs) based on the liver data.

For the calculation of the RBAs, the bioavailability of the orally dosed lead acetate is required. As this resulted in very different values in the two *in vivo* experiments (13 and 7%), we used the results of both experiments to calculate the RBAs.
First, the RBAs were calculated using the bioavailability of the single oral dose (150 mg/kg/d) of lead acetate from the validation study (7%). However, it appeared that most resulting RBAs were higher than 100% (see Table 12), which was considered highly improbable. In the second approach, the RBAs were calculated using the bioavailability of the orally dosed lead acetate from the pilot study, using the equation depicted in Figure 7. Table 12 shows that RBAs based on this second approach are much lower than those based on the validation study, due to the larger bioavailability (AUC) of orally dosed lead acetate in the

pilot (13%). The value of 12% is close to the bioavailability of orally dosed soluble lead in swine in the study of the US EPA (2007), which equalled 15%. For these reasons, in the remainder of this report we will work with the RBA values determined with the dose-response curve of the pilot study (second approach) rather than the RBAs based on the lead acetate data in the validation study.

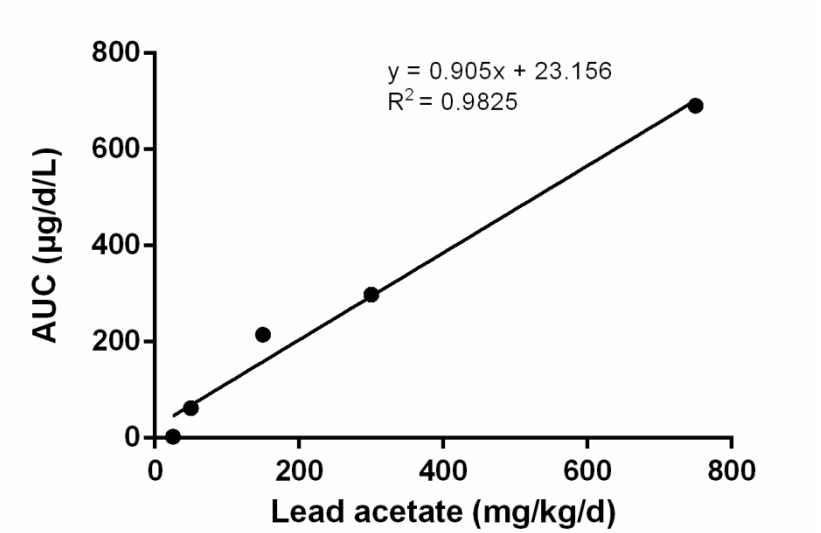

Figure 7 Dose-response curve for blood for orally dosed lead acetate in pilot study (corrected for background).

The influence of the amount of soil on the bioavailability of lead from soil was examined by splitting the Utrecht soil and testing two amounts of soil. Table 12 shows that a lower amount of Utrecht soil resulted in a higher bioavailability determined in blood. A possible cause is that saturation is reached in the swine treated with 1 g soil and not all the lead that is accessible can be dissolved.

Table 12. Calculated bioavailability of lead in soil relative to that of orally dosed lead acetate (RBA): two approaches

Location	First approach: RBA based on validation study[a] (%)	Second approach: RBA based on pilot study[b] (%)	RSD[c] (%)
De Rijp	104	58	28
Leiden	105	59	33
Maastricht	85	47	31
Nijmegen	150	84	15
Rotterdam	100	56	26
Utrecht - 0.4	171	95	12
Utrecht - 1	86	48	2
PbAc (oral)[d]	7	13	

[a] BA of soil (AUC_{soil}/Dose) divided by BA of single oral dose of lead acetate (AUC_{PbAc}/dose) from validation study

[b] BA of soil (AUC_{soil}/Dose) divided by BA of orally dosed lead acetate (AUC_{PbAc}/dose) from pilot study. The BA of PbAc was calculated with the dose-response curve obtained in the pilot study (AUC = 0.905 x dose +23.2)

[c] RSD is the same for both methods

[d] BA of PbAc compared to the intravenously dosed PbAc (AUC_{oral}/dose divided by AUC_{IV}/dose)

6.5 *In vitro versus in vivo*

Figure 8 shows the correlation of the *in vivo* RBAs (second approach, see section 6.4) for blood with the RBAc of the IVD model, Tiny-TIM system and UBM model. The best correlation with *in vivo* RBAs was observed for UBM (R^2=0.80) and for Tiny-TIM (R^2=0.67), while those for IVD (R^2=0.15) and $UBM_{gastric}$ (r^2=0.45) were low. Note that the correlations may be (mainly) determined by the most extreme data points and that the variation in the RBAs is only a factor of 2.

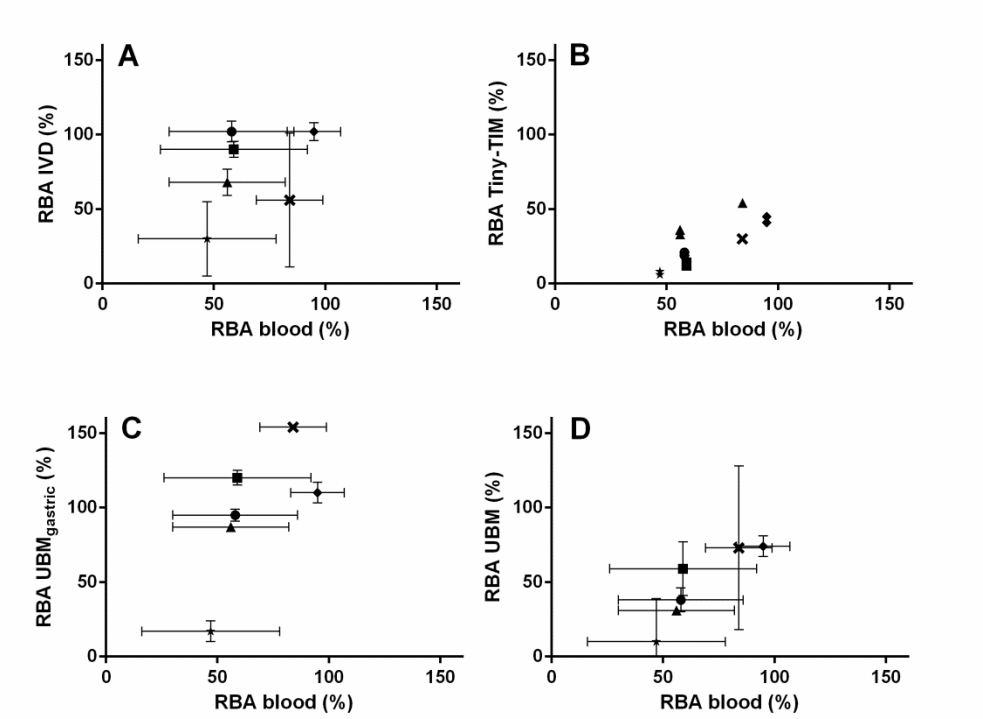

Figure 8 Correlation of the RBA from blood with the RBAc from a) IVD, b) Tiny-TIM system, c) UBM gastric phase and d) UBM gastric + intestine phase. Experiments with Tiny-TIM were performed in duplicate; both values are presented.

RBAs

In Table 13, an overview is given of the measured RBAs in the different models. The RBAs from blood are close to the results from IVD and UBM, whereas the RBAcs from Tiny-Tim and $UBM_{gastric}$ are lower and higher than the *in vivo* data, respectively.

Table 13. Overview of RBAs (%) and RBAcs (%) from in vivo and in vitro studies

Location	Blood	IVD	Tiny-TIM	$UBM_{gastric}$	UBM
The Hague	ND	77	28, 37	84	43
De Rijp	58	102	21, 19	95	38
Leiden	59	90	14, 12	120	59
Maastricht	47	30	6, 8	17	10
Nijmegen	84	56	30, 54	154	73
Rotterdam	56	68	36, 33	87	31
Utrecht	95	102	45, 41	110	74

6.6 Correlation of *in vivo* RBAs with soil extracts (diluted HNO_3)

In addition to the correlation with *in vitro* models, the *in vivo* RBAs are compared to HNO_3 extraction data that may simulate the bioaccessibility. The Pb concentrations extracted with diluted HNO_3 correlate well with the RBA for blood (R^2 is 0.83). In Figure 9, the Pb concentrations are plotted against the RBAs calculated with the 'first' method.

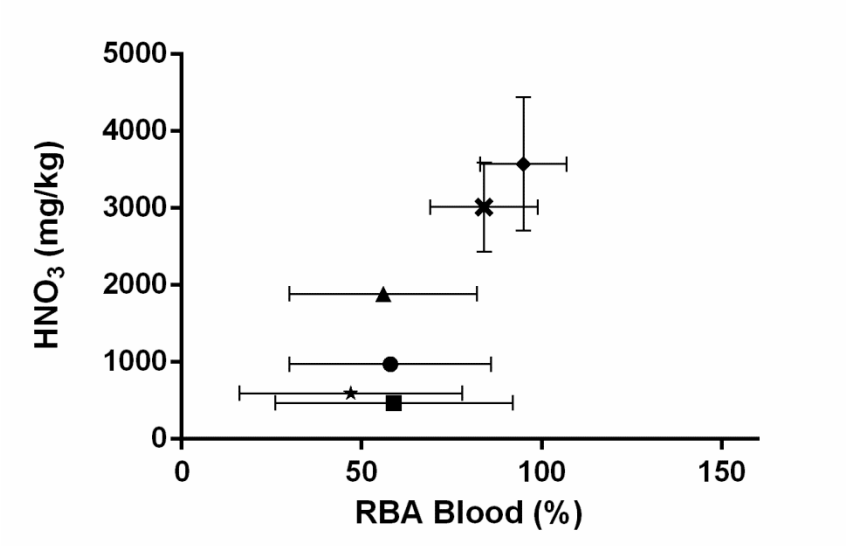

Figure 9 Pb concentrations (mg/kg) obtained from extraction of the test soils with 0.43 M HNO_3 plotted against the RBAs of the test soils, calculated with the 'first' method (Section 6.4)

Because the blood and liver data correlate well, the HNO_3 data also correlate well with the liver data (data not shown).

7 Influence of soil characteristics and lead speciation on RBA

7.1 Introduction

One of the findings with historically contaminated soils was that the bioavailability of lead is affected by the soil characteristics and lead speciation (Oomen et al., 2006). This implies that information on the bioavailability of lead from soil is site-specific. However, it also suggests that the bioaccessibility of lead from soil may be predicted from information on the lead and/or soil characteristics. It may therefore be possible to derive general information on the bioaccessibility and relative bioavailability of lead from soil for specific soils and/or lead types.

Hagens et al. (2009) studied the relationship between soil characteristics and bioavailability in 90 made grounds from the Netherlands. They observed no relation between the release of lead from made grounds in the *in vitro* tests and soil characteristics. According to Hagens et al. (2009) this was possibly caused by the uniformity of the soil characteristics of the studied soils. Although Hagens et al. (2009) did not find any relationship, the influence of soil characteristics and lead speciation on RBA was investigated again in this study. Since the sample sites in this study are selected on the basis of differences in soil characteristics and the fact that the methodology of the *in vitro* methods has changed substantially (see Section 5.1), re-examination was justifiable.

In the present study, the lead content and general soil composition of the samples was determined. The lead source, primary and secondary lead phases[3], and the chemical composition and size of the lead phases in the samples, were already determined by Hagens et al. (2009). Note that these analyses were performed in other samples, but originating from the same made ground sample locations and consequently the composition of the samples may be different. Nevertheless, we assume that in general the samples of the two studies are comparable with respect to lead source and lead phases.

In the current chapter, statistics (Statistica software package) were used to determine if soil and lead characteristics of the made ground have an effect on the (measured) relative oral bioavailability.

7.2 Correlating soil characteristics with relative bioavailability

The present section investigates whether the relative oral bioavailability is a function of several soil characteristics including soil pH, carbonate content, organic matter content, clay content, and lead content for the made grounds.

[3] The mineralogy of lead that entered the soil (primary lead phase) can dissolve over time and form secondary lead containing minerals.

7.2.1 *Regression analysis on soil characteristics*

Regression analyses were performed to see whether the relative oral bioavailability (see Table 12) correlated with the soil characteristics (see Table 1); each soil property tested separately. The resulting plots (presented in Appendix 4) are summarized in Table 14, showing that the lead content (*aqua regia* digestion) of the soil samples shows a slightly positive correlation with the *in vivo* relative oral bioavailability data (blood) and with the Tiny-TIM *in vitro* model and HNO_3 leach. A slight negative correlation is observed for the calcium carbonate content and soil pH of the soil samples and the relative oral bioavailability as obtained with the IVD *in vitro* model. The organic matter content of the soil samples shows a slight negative correlation with the *in vivo* relative oral bioavailability data (blood), the UBM *in vitro* models and the HNO_3 leach. For the clay content, no clear correlations are observed for any the models.

7.2.2 *Multiple regression analysis*

Correlating soil characteristics to RBA

To investigate if multiple soil characteristics can be used to estimate the relative bioavailability of lead in made grounds, a multiple linear regression model is applied. Schematically, this model looks like equation 6 (Hagens et al., 2009):

$$\log(RBA) = a\times\log(\textit{calcium carbonate}) + b\times\log(\textit{organic matter}) + c\times\log(\textit{clay}) + d\times\log(\textit{total Pb}) + \varepsilon \quad [6]$$

where
RBA = relative bioavailability from *in vivo* study
ε = residue (= intercept)

Since the number of observation is small (n=6), the number of independent variables (calcium carbonate, organic matter, clay and total Pb) that can be tested is restricted to three. For this reason, since soil pH and calcium carbonate content are correlated, it was decided not to include soil pH in the multiple linear regression model.

Based on the limited number of observations it was not possible to determine the distributions (normal, lognormal or other) of the variables. Nevertheless, Hagens et al. (2009) applied a log transformation to the variables in equation 6 based on 90 soil samples from made grounds, since the variables' distributions were highly skewed to the right. The residue ε is assumed to have a normal distribution.

The predictive multiple linear regression model was optimized and run (Statistica software package) to determine which variables are related (and their significance) to the relative oral bioavailability of lead. The statistical results of the fitted model are summarized in Table 14.

Table 14. Correlation of RBA with soil characteristics: Statistical results (a, d, c, d, ε and P-values in brackets) of the single linear regression model. Significant coefficients (P<0.05) are indicated with ###.

RBA	a: log($CaCO_3$)	b: log (OM)	c: log (clay)	d: log(Pb)	ε
log(Blood)	-0.109 (P=0.16)	-0.496 (P=0.06)	-	0.305 (P=0.06)	1.446 (P<0.05) ###
log(TIM)	-0.370 (P=0.20)	-1.075 (P=0.14)	-	0.869 (P=0.07)	-0.410 (P=0.69)
log(IVD)	-0.486 (P<0.05) ###	-0.705 (P<0.05) ###	-	0.251 (P<0.05) ###	1.786 (P<0.05) ###
log($UBM_{gastric}$)	-0.639 (P<0.05) ###	-1.566 (P<0.05) ###	-	0.656 (P=0.08)	1.267 (P=0.19)
log(UBM)	-0.574 (P<0.05) ###	-1.688 (P<0.05) ###	-	0.654 (P<0.05) ###	1.017 (P=0.10)
Log HNO_3	-0.171 (P<0.05) ###	-0.501 (P<0.05) ###	-	0.344 (P<0.05) ###	1.315 (P<0.05) ###

As can be seen in Table 14, clay content is not found to be a predictive variable for the relative bioavailability of lead. The other variables – lead content, carbonate content and organic matter content – seem to some extent predictive for the relative bioavailability of lead. The results in Table 14 suggest that the relative oral bioavailability of lead increases with an increasing lead content and decreases with an increasing calcium carbonate and increasing organic matter content. For log(IVD), log(UBM) and log(HNO_3) a significant correlation (P<0.05) with several soil properties is observed. For log(IVD), log(UBM) and log(HNO_3) a significant correlation is observed with log($CaCO_3$), log(OM) and log(Pb), whereas for log($UBM_{gastric}$) a significant correlation is observed with log($CaCO_3$) and log(OM).

In Appendix 5 the measured (observed) relative oral bioavailability of lead – as obtained with the *in vivo* and *in vitro* methods – is plotted versus the predicted relative oral bioavailability of lead as obtained with the model parameters in Table 14. Based on these results it appears that the relative oral bioavailability of lead can be partly predicted based on the soil characteristics, Pb content, calcium carbonate content and organic matter content. It is however noted that the number of observation is low and the results are therefore indicative. Model validations are recommended.

Prediction of *in vivo* bioavailability with *in vitro* models

Figure 8 (see Section 6.5) shows that the relative bioavailabilities of lead derived with the *in vivo* methods do not correlate well with the *in vitro* results. To determine if the observed differences between the *in vivo* and *in vitro* methods are caused by soil characteristics, a second multiple linear regression analysis was performed. The used model is comparable with equation 6, with log(*rel*

bioavailability) of the *in vitro* methods as extra independent variables and log(*rel bioavailability*) of the *in vivo* method (only blood) as dependent variable (see equation 7). The statistical results of the fitted model are summarized in Table 15.

$$\log(RBA_{blood}) = a\times\log(calcium\ carbonate) + b\times\log(organic\ matter) + c\times\log(clay) + d\times\log(total\ Pb) + e\times\log(RBAc_{in\text{-}vitro}) + \varepsilon \quad [7]$$

where

RBA_{blood} = relative bioavailability based on blood data *in vivo* study

$RBAc_{in\ vitro}$ = relative bioaccesibility based on *in vitro* data

Table 15. Statistical results (a, d, c, d, e, ε and P-values in brackets) of the multiple linear regression model (see equation 7). Significant coefficients (P<0.05) are indicated with ###.

RBA	**a: log ($CaCO_3$)**	**b: log (OM)**	**c: log (clay)**	**d: log (Pb)**	**e: log (in-vitro RBA)**	**ε**
log(Blood) dependent var. **(log(TIM** independent var.)	-	-0.287 (P=0.12)	-	-	0.249 (TIM) (P=0.06)	1.874 (P<0.05 ###
log(Blood) dependent var. **(log(IVD)** independent var.)	0.305 (P<0.05) ###	-	-	-	0.855 (IVD) (P<0.05) ###	0.289 (P=0.30)
log(Blood) dependent var. **(log(**$UBM_{gastric}$**)** independent var.)	-	-0.268 (P=0.27)	-	0.216 (P=0.19)	0.110 ($UBM_{gastric}$) (P=0.40)	1.302 (P=0.08)
log(Blood) dependent var. **(log(**UBM**)** independent var.)	-	-0.179 (P=0.35)	-	0.193 (P=0.15)	0.175 (UBM) (P=0.19)	1,240 (P=0.05)
log(Blood) dependent var. **(log(**HNO_3**)** independent var.)	0.052 (P=0.22)	-	-	-	0.917 (HNO_3) (P<0.05) ###	0.176 (P=0.58)

As can be seen in Table 15, clay content is not a predictive variable for the observed difference between the relative bioavailability of lead in blood and the *in vitro* methods. The other variables – lead content, carbonate content, organic matter content, and relative bioavailability (*in vitro* methods) – seem to some extent predictive for the observed differences between relative bioavailability of lead determined in blood and with the *in vitro* methods. For the difference between relative bioavailability in blood and the HNO_3 model, a significant correlation with log(RBAc)-HNO_3 is observed. Only for the difference between relative bioavailability in blood and the IVD model, a significant correlation with a soil characteristic (log($CaCO_3$)) and log(RBAc)-IVD is observed. These results suggest that the observed difference between RBA-blood and RBAc-IVD is mainly caused by the calcium carbonate content of the soil samples. Possibly calcium carbonate binds lead better in the IVD model than *in vivo* in swine. Another explanation might be that the buffering capacity of calcium carbonate influences the pH of the *in vitro* models in a different way than *in vivo*. However, this is not very likely, as the amounts of NaOH added and the pH-values measured during the *in vitro* experiments for the soils high on calcite did not deviate from those of the soils with a low fraction of calcite.

Although the RBAc-values from the IVD model appear to predict the *in vivo* RBA-values well using this regression model, it should be emphasized that the number of observations is limited and that validation of this regression model is recommended.

The observed difference between RBA-blood and RBAc-Tiny-TIM and RBAc-$UBM_{gastric}$ and RBAc-UBM can only be explained by a combination of factors (log(OM), log(Pb) and log(RBA), of which none is significant. It is remarkable that the fraction of calcite appears not to play a role in these *in vitro* methods, in contrast with IVD (significant) and HNO_3 (non-significant). Again, it is noted that the number of observations is limited.

In Figure 10 the measured (observed) relative oral bioavailability of lead – as obtained with the *in vivo* blood method – is plotted versus the predicted relative oral bioavailability of lead as obtained with the *in vitro* method, taking into account the possible influence of soil properties on the RBA as listed in Table 15. Although high correlation coefficients are observed (R^2=0.79-0.95), all results are indicative due to the limited number of observations. However, as the models were only tested with the same samples as were used to build the model with, they should be validated with independent samples.

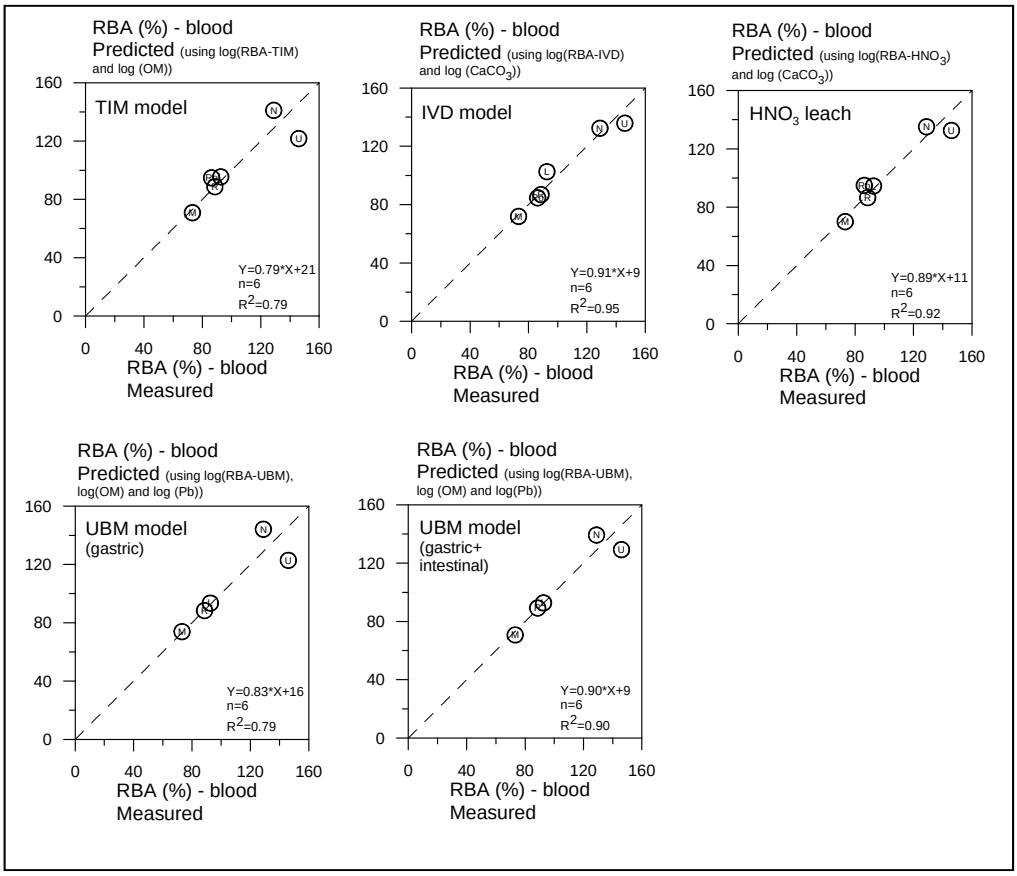

Figure 10 Measured relative oral bioavailabilityof lead in blood (expressed as %) versus the predicted relative oral bioavailability of lead (expressed as %) as obtained with the in vitro method taking into account the possible influence of soil properties. The solid line represents the 1:1 line. Sample locations are: D=The Hague, L=Leiden, M=Maastricht, N=Nijmegen, R=De Rijp, Ro=Rotterdam, U=Utrecht.

7.3 Correlating soil and anthropogenic lead characteristics with relative bioavailability

7.3.1 Introduction

Previous studies on the bioavailability of lead from soil indicated that the bioavailability of lead can be influenced by:

1. The chemical composition of the anthropogenic lead source and its solubility (Steele et al., 1990; Cotter-Howells et al., 1991) (Davis et al., 1993; Ruby et al., 1992; Ruby et al., 1996; Ruby et al., 1999) (Rieuwerts et al., 2000; Hettiarachchi et al., 2004).
2. The specific reactive surface of lead in soils (Steele et al., 1990; Ruby et al., 1992; Ruby et al., 1999).
3. The soil type, and capacity to form secondary lead phases (Casteel et al., 1997; Rieuwerts et al., 1998a; Rieuwerts et al., 1998b; Rieuwerts et

al., 2000; Ruby et al., 1999; Yang et al., 2003; Hettiarachchi and Pierzynski, 2004).

Figure 11 provides a schematic overview of these processes that are believed to control the bioavailability of lead in soil (Ruby et al., 1999). Different lead forms exhibit different rates of lead dissolution, depending on their chemistry and particle size distribution, the mechanism by which they dissolve (e.g. surface reaction or transport-controlled dissolution kinetics), and the geochemistry of the soils in which they are present. This indicates that the bioavailability of lead from soil may be predicted based on information on the lead characteristics. The objective of this part of the research is to determine if relative oral bioavailability of lead is related to lead characteristics.

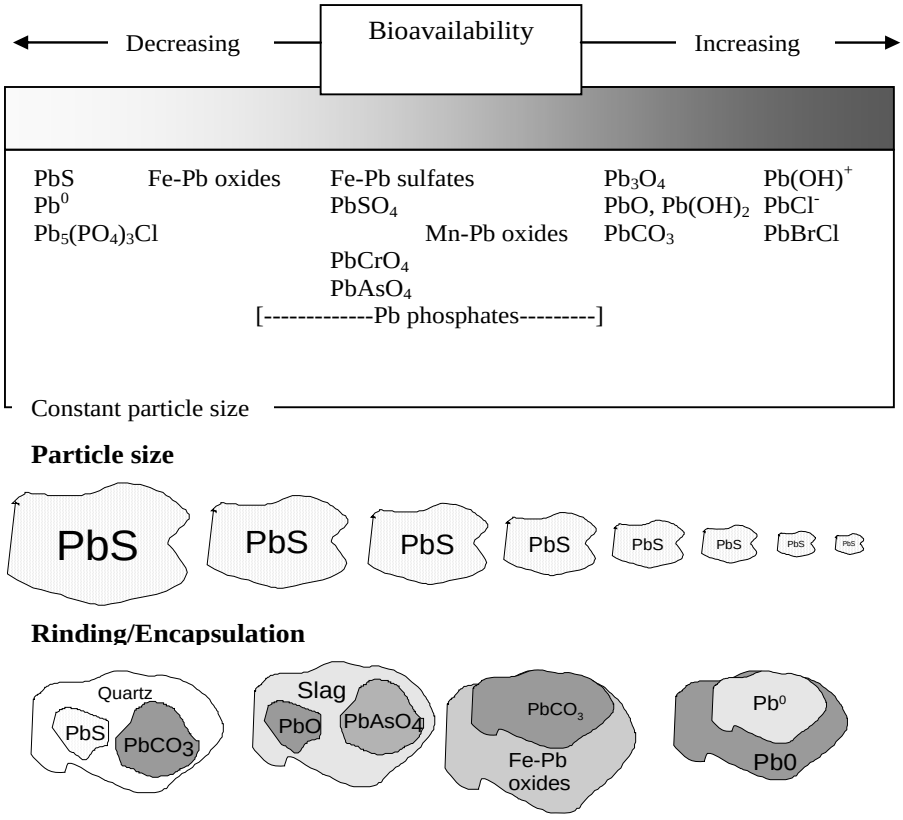

Figure 11 Schematic overview of how different lead species, particle sizes, and morphologies affect lead bioavailability (after Ruby et al. (1999), with permission).

7.3.2 *Grouping of made grounds by anthropogenic lead characteristics*

Hagens et al. (2009) already determined the anthropogenic lead characteristics of made ground samples from De Rijp, Maastricht, Nijmegen en Leiden. The methods used to determine the characteristics of anthropogenic Pb are described in detail in Hagens et al. (2009). The results of this study are summarized in Appendix 4. The anthropogenic lead characteristics of the Dutch made grounds from Utrecht, The Hague and Rotterdam – sampled in this study – are not determined. However, the Utrecht made ground sample most likely contains lead white, because the sample location was situated close to a former lead white factory.

Based on the available data on lead characteristics the made grounds can be divided into 3 groups:

1) Soil mainly polluted with lead glass or lead glaze (De Rijp, Leiden and Maastricht). The diameter of these primary phases is relatively large (up to 675 μm). These samples contain no to very few secondary lead phases and the lead content of organic matter particles is very low. It is concluded that the solubility of these primary minerals is relatively low, due to the small reactive surface and the incorporation of lead in a glass matrix. This low solubility resulted in the formation of no to very little secondary lead phases.
2) Soil mainly polluted with lead, lead oxide or lead carbonate (Nijmegen). The diameter of these primary lead phases is relatively small. The mean lead content of organic matter in these samples is relatively high and the

soil contains lead apatite minerals. It is concluded that the solubility of these primary lead phases is relatively high, due to the large reactive surface and the presence of the substantial amount of secondary lead phases. This is confirmed by the dissolution holes in the primary lead phases in the Nijmegen soil (Hagens et al., 2009). The newly formed secondary lead phases are (under the prevailing soil conditions) less mobile than the primary lead phases. The studied made ground from Utrecht most likely belongs to this group (lead from lead white factory).

3) Soil from which the lead characteristics are not studied (The Hague and Rotterdam).

7.3.3 PPS-ranking

To determine if lead mineralogy and particle size are related to the relative bioavailability of lead, the anthropogenic Pb characteristics of 5 soil samples (De Rijp, Leiden, Nijmegen, Maastricht and Utrecht) are ranked based on the primary lead phases present (P), the particle size (P) and the secondary lead phases present (S). Although the relative oral bioavailability of lead might be predicted based on the measured soil characteristics (see Section 7.2), these were not taken into account in the classification, because these results are only indicative and not validated yet. This ranking system was developed by (Hagens et al., 2009) and is presented in Appendix 4. The results of the PPS ranking vary from 2 to 7 (Table 16). The soil sample from De Rijp revealed the lowest PPS ranking (2), which is due to the presence of large particles of lead glass/glaze. This low PPS ranking number corresponds to a relative low RBA_{blood} of 89%. It is predicted that the soil sample form Utrecht will have the highest PPS ranking (7). This is due to the presence of small particles of lead white. Indeed, this soil sample shows the highest RBA_{blood} (146%).

Table 16. The results of the PPS ranking and the corresponding RBA_{blood} for the locations of the made ground samples.

Sample location	**P** Primary Pb phases	**P** Particle size	**S** Secondary Pb phases	**PPS** Ranking	**RBA** blood
De Rijp	1	1		2	89
Leiden	1	4		5	93
Nijmegen	3	2	-1	4	129
Maastricht	1	3		4	73
Utrecht	3 (predicted)	4 (predicted)	nd	7	146
Rotterdam	nd	nd	nd	nd	86
The Hague	nd	nd	nd	nd	nd

nd= not determined

A slightly positive correlation between the relative oral bioavailability for lead (RBA), as determined with the *in vivo* and the *in vitro* models and the PPS ranking was found (see Appendix 4). However, based on the limited number of samples (n=4-5) and the qualitative nature of the arbitrary PPS ranking, no firm conclusions can be drawn on these relationships. Currently, it is only possible to predict whether bioavailability values will be low, medium or high, based on the

anthropogenic lead characteristics. Nevertheless, this information could help in determining remediation strategies and priority ranking of made grounds which are polluted with lead. This information also helps in understanding the factors that affect relative oral bioavailability of lead from soil.

8 Discussion

In the first section of this chapter, the results of the *in vitro* experiments are discussed, also in relation to previous studies. In the second section, the findings of the *in vivo* study are considered and compared with those of the *in vitro* models. The different models (*vitro* and *vivo*) are evaluated by comparing the *relative* bioaccessibilites (or in case of *in vivo* results, relative bioavailabilities). The last section addresses the implication of the results of this study for the risk assessment of lead in Dutch made grounds. This includes an assessment of lead based on the *absolute* bioavailability to children, in order to derive the relevant exposure of lead to children.

8.1 *In vitro* models

8.1.1 IVD model

Selection of best set-up

The pilot experiments with the IVD model demonstrated a better reproducibility using 0.2 g soil than using 0.06 g. The RSD for the runs with 0.2 g soil, without the addition of food, is < 10%, which is below the bench mark criterion (of < 10%) as proposed by Wragg et al. (2011). Adding food to this system increased the RSD up to 15%, while the use of 0.06 g soil yielded a RSD up to 22%. In the UBM 0.6 g soil is used per digestion tube and for this reason obtains a better reproducibility. Nevertheless, it was shown in previous experiments (Oomen et al., 2006) that this amount of soil reduces the bioaccessibility in the IVD model most likely due to the solid-to-fluid ratio. This appeared not to be the case with 0.2 g of soil in the pilot experiments. The best reproducibility was found for the upscaled model, without adding food (RSD < 5%). Nevertheless, this model appeared very labour-intensive and for this reason, the model with 0.2 g (without adding food) was selected for use in the validation study.

In contrast to the pilot study, indicating that 0.2 g soil does not reduce the bioaccessibility, the validation study showed a different outcome. For the sample locations Nijmegen and Maastricht (not tested in the pilot study), both with a high calcite fraction, this amount of soil appears to affect the bioaccessibility without affecting the gastric pH. For this reason, for future use of IVD, for calcite-rich soils the use of 0.06 g rather than 0.2 g soil is recommended, combined with a large number of replications (e.g. eight). The latter are necessary to reduce the uncertainty due to large variation because of possible inhomogeneity of the soils. Another possibility may be to correct the observed bioaccessibilities with the mathematical model for calcite presented in Chapter 7. However, this mathematical correction model has not been validated.

Although the results of the pilot experiments showed a good reproducibility, that of the validation samples is sometimes much greater than 20%, up to 45%. This is likely due to inhomogeneity of the Dutch made ground samples, since the variation in the homogeneous reference Montana soil was only 1%. It was decided not to grind the soil to more homogeneous samples, as this could influence the bioaccessibility, and to use a fraction of < 2 mm rather than < 250 µm (as is internationally used), since the former size fraction is according to Dutch legislation (Dutch standard NEN5709:2006). In the previous study (Hagens et al., 2009), in which the soils were first milled and then sieved, a

variation of 20% in the duplo bioaccessibility measurements with IVD (0.06 g soil) was obtained.

Lead acetate
While the results for the bioaccessibility of lead acetate in the first pilot experiment with the IVD model appeared reliable, in the second pilot the results for the bioaccessibility of this reference substance were lower than expected, with values ranging from 8% to 30% bioaccessibility. In the validation study, the results for lead acetate were even lower, whereas those for the reference soil (Montana) were stable (average bioaccessibility of 18%, 14% and 24% for the three experiments). The reason for these inconsistent findings for lead acetate is unclear. For the calculations of the relative bioaccessibility measured with IVD, the data on lead acetate of the first pilot were used.

8.1.2 Tiny-TIM

Amount of soil added
The pilot study with Tiny-TIM has revealed a large impact of soil amount on the bioaccessibility of lead. The amount of 0.5 g soil resulted on average in a 5-fold higher bioaccessibility compared to the traditional use of 5 g soil. Apparently, when 5 g of soil is added to Tiny-TIM (ratio solid: liquid of 1:51) (Hagens et al., 2009), the system is oversaturated and not all the 'potentially' bioaccessible lead can dissolve. Reduction of the amount of soil to 0.5 g leads to a solid: liquid ratio which is comparable to that of IVD (1:300).

Recoveries
During the experiments, recovery was measured for three soil samples (n=1) and PbAc (n=2). While recoveries for PbAc were good (93 ± 3%), recoveries found for the test soils were 55% (The Hague), 75% (Rotterdam) and 292% (Nijmegen). As the destruction of the residues in Tiny-TIM is done with *aqua regia* with the same method used for the test soils, the binding of lead to soil in the TIM system is not a very likely explanation for the low recovery results. The recoveries differing from 100% are probably due to inhomogeneous soils that lead to incorrect estimates of the total lead in the system, especially for Nijmegen (soil with crude pieces). Note that when the total lead in the system is not correctly estimated, this also influences the RBA. This has to be kept in mind, especially for the Nijmegen measurement.

8.1.3 Differences between IVD and Tiny-TIM explained

The difference between the results of IVD and Tiny-TIM determined in Hagens et al. (2009) was thought to mainly originate from differences in pH of the gastric phase and the difference in separation technique. Based on these data both *in vitro* models were modified to more closely mimic the semi-fed child having hand-to–mouth contact and to investigate the influence of certain parameters on the outcome of the models. For this reason, the stomach pH of Tiny-TIM in the first 120 minutes was lowered: from pH 5 to 2 in the previous study to pH 2.8 to 1.7 in in the present one. Secondly, as described above, the smaller amount of 0.5 g soil was tested in Tiny-TIM, simulating hand-to-mouth rather than pica behaviour (eating of soil). The resulting higher bioaccessibilities partially explain the different outcomes between the IVD and Tiny-TIM model in the study by Hagens et al. (2009).
Furthermore, it appeared that the RBAcs determined with Tiny-TIM, when Pb amounts in the centrifuged intestinal chyme and in the dialysate are summed, are fairly comparable with the RBAcs of the IVD model. This demonstrates that

the separation technique is another important cause of the different outcomes of the two models.

The large influence of the different separation techniques can be partly explained by the type of fraction yielded by each technique. Centrifugation of the chyme (as in IVD) is used to simulate the bioaccessible fraction in the intestine (F_B= bioaccessible fraction, which equals the *maximum* amount that can be absorbed). The centrifugated chyme contains free lead ions, and lead that is (reversibly) bound to complexes with large proteins. The latter cannot pass the intestinal wall, but the reversibly bound lead ions may dissolve and then pass. In Tiny-TIM, the contents of the intestinal compartment are passed through a membrane, thereby excluding lead complexes with proteins ≥ 10 kDa. For this reason, the dialysate of Tiny-TIM mimics the fraction that is passively absorbed over the intestinal wall (F_B x F_A, bioaccessible fraction x the absorbed fraction). Taking this into account, and calculating F_B x F_A using the IVD results (assuming F_A= 0.8), the difference between the RBAc as determined by IVD and Tiny-TIM in Hagens et al. (2009) was, on average, a factor of 5 and in the present study it is a factor of 3.

8.1.4 UBM compared with the other two in vitro models

The UBM model was the only model tested for the gastric phase as well as the gastric and intestinal phase. The latter yielded lower RBAcs by a factor of 1.5-3, due to the higher pH in the intestinal phase. As expected, the bioaccessibility of PbAc was also lower in the intestinal phase (66%) than in the gastric phase (99%). In the study by Denys et al. (2012), the difference in relative bioaccessibilities between the two phases of the model was found to be small. On the other hand, Oomen et al. (2006) found absolute BAcs in the gastric phase a factor of about 2 higher than BAcs of intestinal phase (number of soils: 11), while Koch et al. (2013) determined a factor of 3 difference for Montana soil. The ratio between absolute bioaccessibility of the two phases in the present study varies from 2 to 4.

When comparing the results of UBM with those of IVD, it should be kept in mind that the conditions were different. UBM used fasted conditions in combination with 0.6 g of soil and a centrifugation rate of 4500 g, while the IVD conditions were semi-fed, 0.2 g of soil and 2900 g. In addition, the samples of IVD and UBM were analysed in different laboratories. While a fasted model would lead to a higher bioaccessibility, a greater amount of soil and a higher centrifugation rate would result in a lower one. From experiments with the IVD model, it is known that an amount of 0.6 g soil (solid: liquid of 1:100) is so high that it reduces the bioaccessibility of lead in the model (Omen et al. 2006). Since the RBAcs for the UBM model are lower than those of IVD by a factor of 0.8[4]–3, it may be concluded that the combined effect of the greater amount of soil and higher centrifugation rate in UBM was stronger than the effect of the fasted conditions. This supports earlier findings: Hagens et al. (2009) showed that for 70 soils the average ratio fed/fasted was 0.65 (P95 was 0.97), while the ratio between RBAcs determined with 0.06 g and 0.6 g varied from 1-9 (Oomen et al., 2006). Note that the solid: liquid ratio of 1:1000 is comparable with hand-to mouth contact (Bakker and Hagens, 2010).
The single regression analysis showed that RBAcs of UBM, in contrast with those of IVD, did not correlate with the calcite fraction in the soil. Apparently, the UBM

[4] for Nijmegen UBM yielded a higher RBA than IVD

model was not as sensitive to high calcite fractions in the soil as IVD, despite the higher amount of soil added to the system. The UBM model only showed a relatively low RBAc for the soil from Maastricht. This may be explained by saturation caused by the high amount of salts being dissolved from the sample, therefore not allowing full dissolution of lead.

8.1.5 Measured bioaccessibilities compared with previous studies

The bioaccessibilities of the seven made grounds measured with IVD were compared to the values determined for the same locations in the previous study (Table 17). The values presented by Hagens et al. (2009) were determined under fasted conditions and corrected by the authors for the average physiological state with the use of a correction factor. In the present study, the IVD model was adapted to a semi-fed state by averaging the pH and the juice compositions of the fed and fasted state, and 0.2 g of soil was used rather than 0.06 g. Even with this difference in method, the results from the two studies are comparable for most locations, except for De Rijp and, to a lesser extent, Leiden.

Table 17. Bioaccessibility (BAc) of the soil samples determined by the IVD model for the same locations in the present and previous study

Sample	Present study BAc semi-fed (%) n = 4	Hagens et al. (2009) BAc_{APS} (%) n =2
The Hague	45 ± 42	50
De Rijp	60 ± 7	21, 23
Leiden	53 ± 5	25, 34
Maastricht	18 ± 25	21, 25, 68
Nijmegen	33 ± 45	20, 23
Rotterdam	40 ± 9	47, 47
Utrecht	60 ± 6	67

APS: Average Physiological State: bioaccessibility values were determined under fasted conditions and corrected for APS using a correction factor (Hagens et al., 2009).

To see whether the present samples are representative for Dutch made grounds in the Netherlands, the bioaccessibilities of the seven made grounds measured with IVD are compared to the 90 values determined in the study by Hagens et al. (2009) (Table 18). There is less variation in the BAcs of the present study. Furthermore, the average, P50, P80 and P90 of the present study are all higher than in the previous study, although the difference is not very large (maximum a factor of 1.3). It is not clear whether this is caused by the selection of the sample locations or by systematically higher measurements in the present study.

Table 18. Bioaccessibility (BAc) of made grounds measured by Hagens et al. (2009) and in the present study

	Hagens et al. (2009) n = 90 BAc_{APS} (%)	Present study n= 7 BAc semi-fed (%)
Average	36	44
Lowest value	5.4	12
Highest value	88	72
P50	34	47
P80	46	60
P90	51	63

APS: Average Physiological state: BAc values were determined under fasted conditions and corrected for APS using a correction factor determined in (Hagens et al., 2009).

8.2 *In vivo* study

8.2.1 Effect of amount of soil

The Utrecht soil was fed to juvenile swine in two different doses (3 pigs for each dose) to investigate the potential effect of the amount of soil consumed on the bioavailability of lead. Increasing the amount of soil from 0.4 to 1 g/kg bw/d led to a decrease in bioavailability of 30% in the liver and of 50% in blood. The highest dose corresponded with a total lead concentration of 2800 µg/kg bw/day. This concentration of lead was outside the dose range that displayed a linear dose-response relationship in the pilot study (up to 800 ug/kg bw/d). Apparently, at high dose levels, such as 2800 µg/kg bw/day, the dose-response relationship is no longer linear due to saturation of lead in blood and liver of the animals. The bioavailable lead concentrations of the other soils dosed with 1 g/kg bw/d were within this linear range.

8.2.2 In vivo-in vitro correlations

The R^2s of the correlation of the RBAcs measured *in vitro* and the RBAs from the *in vivo* data increased in the order of IVD < $UBM_{gastric}$ < Tiny-TIM < UBM. The R^2s of Tiny-TIM and UBM agree with the benchmark criterion given by Wragg et al. (2011) (R^2 should be greater than 0.6). Note that the correlations are independent of the method used to calculate the RBAs.

Several differences in the experimental set-up between the *in vivo* study and *in vitro* studies may have influenced the correlations found:

- *In vivo*, the soil was dosed via a small portion of feed, while *in vitro* the food matrix was absent. As the pH is well stabilized *in vivo*, the influence of the portion of feed on the bioavailability of lead is expected to be small.
- *In vivo* the dose was given once a day for 7 days, while *in vitro* the dosing was only once. Repeated dosing was performed to obtain measurable amounts in swine blood and tissue and is not expected to influence the bioavailability.
- The *in vitro* models were 'tuned' to a semi-fed child (except for UBM, which mimicked a fasted child), while the test animals were semi-fed young pigs (modelling semi-fed young children). Although swine are considered a good model for children according to the US EPA (2007), lead absorption in juvenile swine (± 15%) is lower than for young children (42-53%). Although the reason for this difference is not known, it is important to note that even if swine do absorb less lead than

children under similar dosing conditions, this does not invalidate the swine as an animal model for estimating *relative* bioavailability of lead in different test materials (EPA, 2007).

The IVD model gives a better prediction of the RBAs of made grounds when soil and lead characteristics are taken into account as was done with the results of the multi-regression analysis (Section 7.2.2). It appears that especially the presence of calcite should be considered: the calcite-rich (10%) soil from Maastricht shows a low bioaccessibility in all of the *in vitro* models, but a relatively high bioavailability in blood and liver. As the pH in the IVD experiments was not influenced by the addition of the calcite-rich soils, this effect cannot be explained by the saturation of the solution due to less acidic conditions. It still may be a saturation effect caused by the large amount of salts being dissolved from the sample, therefore not allowing full dissolution of lead. Further testing of this soil at a higher liquid to solid ratio would be needed to see if this increases the lead extracted. For now, a clear explanation for these findings cannot be given. The other soil rich on calcite (Nijmegen, 13%) also has higher RBA values *in vivo* than *in vitro*, although the difference is not so striking as for the Maastricht soil. Multiple regression analysis (Chapter 7) showed that when taking the fraction of calcite into account, the bioavailability of lead in made grounds can be predicted well by the IVD model. Nevertheless, since there is no sound explanation for the effect and, in addition, the number of tested soils was small, we will not derive a correction factor for calcite.

Previous comparisons of bioaccessibility results with *in vivo* bioavailability studies of lead from soil in juvenile swine were reported by Hagens et al. (2009). The correlation between bioaccessibility as determined by the IVD model (using 0.06 g soil) and relative bioavailability of lead from soil (n=10) as determined *in vivo* in juvenile swine (Casteel et al., 2006) was fair (R^2 of 0.66), and the slope of the line was close to 1 (1.16) (Oomen et al., 2006).
In a previous study with reference Bunker Hill soil, it was shown that the bioaccessibility of lead in the IVD model overestimated the bioavailability data obtained in the human adults study, especially in the fed state (Maddaloni et al., 1998; Oomen et al., 2006). The results for these soils for Tiny-TIM were comparable with these *in vivo* data (Van de Wiele et al. (2007), (see also Table 9.3 in Hagens et al. (2009)).
UBM was validated by testing 16 soils mostly originating from smelting or mining activities in a study by Denys et al. (2012). RBAs were determined in juvenile swine (liver, kidney, bone and urine) and RBAcs with UBM. *In vivo-in vitro* correlations for lead were good; for lead, regression statistics showed that the slope was around 1 and $R^2 > 0.7$.

Although the extraction of the sampled soils with diluted HNO_3 is not an *in vitro* model, it is striking that the lead concentrations correlate well with the relative bioavailability in the swine ($R^2 = 0.83$ for RBA_{blood}). As this extraction method has been used in ecological risk assessment, it was hypothesized that it may give an indication of the bioavailability to children/swine and this was confirmed in the present study.
In addition, in a recent workshop (7^{th} International Workshop on Chemical Bioavailability 2013, see http://www.bgs.ac.uk/news/events/bioavailabilityWorkshop/), a good correlation between extractions with diluted acids and bioaccessibility was presented by Le Bot (diluted HCl) and Römkes (diluted HNO_3). These unpublished results indicate that the extraction with

diluted HNO_3 may be used to screen soils with respect to their content of bioaccessible lead.

In summary, the *in vitro* models appear able to predict RBAs for lead well for a number of soils, mostly originating from mining and smelter sites. Tiny-TIM and UBM also correlate (fairly) well (R^2=0.67 for Tiny-TIM, R^2= 0.80 for UBM) for the made grounds tested in the present study, whereas the IVD model without calcite correction does not. The suitability of the *in vitro* models for the risk assessment of lead in made grounds is addressed in Section 8.3.

8.2.3 Bioavailability of lead acetate

The bioavailability of orally dosed lead acetate in this study was 7% (validation study, based on one dose) and 13% (pilot study, based on 6 doses), a difference of a factor of 2, which is considered large. There were only two known differences between the two experiments. First, the swine had slightly different ages at the beginning of the experiments (pilot: 7 weeks, validation study: 5 weeks). The second difference is the addition of aluminium salt as a reference material in the validation study, which was included for a parallel study of aluminium in soil (by RIKILT). Nevertheless, this is not expected to influence the behaviour of lead in the gastrointestinal system (note that a similar set-up was used by Denys et al. (2012)).
The RBAs calculated with the bioavailability of lead of the validation study (7%) were almost all higher than one, which is highly unlikely since lead in soils are not expected to be more bioavailable than soluble lead acetate in feed. In addition, the bioavailability of orally dosed lead acetate in the pilot study is close to the value determined by the US EPA (2007). For these reasons, it was concluded that the results for the bioavailability of lead acetate from the validation experiment were not reliable and most likely the result of an unexplained experimental error. Consequently, it was decided to discard the RBA values calculated with these data. The remaining RBA values, based on the bioavailability data of the pilot study, are therefore the only RBA values considered for the risk assessment of children (below).

8.3 Implication for risk assessment of lead in made grounds for children

8.3.1 Bioavailability of lead from made grounds to children

For risk assessment of lead in children, the (site-specific) absolute bioavailability of lead from soil is required. The basic equation for this value from *in vivo* data is as follows (EPA, 2007):

$$ABA_{soil} = ABA_{soluble} \cdot RBA_{soil} \qquad [7]$$

where:
ABA_{soil} = Absolute bioavailability of lead in soil ingested by a child
$ABA_{soluble}$ = Absolute bioavailability in children of some dissolved or fully soluble form of lead
RBA_{soil} = Relative bioavailability of lead in soil from *in vivo* experiment

The RBA_{soil} was determined in the current study. Therefore, to obtain the bioavailability of lead from soils to children, the RBAs of the swine study need to be multiplied with the bioavailability of (dissolved) lead in children. Results from balance studies in infants and young children (age 2 weeks to 8 years) suggest

that lead absorption from the diet is probably 42% to 53% (Alexander et al., 1974; Ziegler et al., 1978). US EPA estimates that the absolute bioavailability of lead from water and the diet is usually about 50% in children (U.S. EPA, 1994). In the derivation of the Dutch Maximum Permissible Risk of lead, a dietary absorption of 40% is used (Baars et al., 2001).

The absolute bioavailability of lead for children as obtained from the six tested soils was calculated by multiplying the RBA ($AUC_{soil}/dose_{soil}$ divided by $AUC_{PbAc}/dose_{PbAc}$) with 47% (realistic value based on range of 40-53%) and is shown in Table 19. The RBAs based on the validation study were omitted, as they were considered to be very unlikely (almost all >100%; see also section 8.2.3). For the Utrecht soil, RBA as calculated from low dosed swine (0.4 g/kg/d) is used.
The ABAs measured in the *in vitro* models are presented in the table as well. It should be noted that the IVD and UBM model estimate the maximum bioaccessibility. For this reason, for comparison with the *in vivo* bioavailability, the bioaccessibility should be multiplied with the absorbed fraction (F_A, see equation 1, assumed to be 0.8). Due to the dialysis membrane, results of Tiny-TIM represent the bioavailability of lead from the soil (and correction for the F_A is not needed). Furthermore, the amounts of lead extracted with HNO_3 as percentage of the total lead determined with *aqua regia* (also multiplied with F_A of 0.8) were included.

Table 19. Absolute bioavailability of lead from soils (%), according to the different models. For the in vivo study these were calculated by multiplying RBA with an absolute availability of dissolved lead of 47%. For the IVD and UBM in vitro studies and for the ratio between lead concentrations in extractions with diluted HNO_3 and aqua regia the ABA as determined with the model was multiplied with a F_A of 0.8.

Location	Vivo, pilot study[1]	Tiny-TIM	UBM	IVD	UBM gastric	0.43 M HNO_3 / *aqua regia*[2]
The Hague	n.d.	15	22	36	66	34
De Rijp	27	9	20	48	75	69
Leiden	28	6	31	42	95	72
Maastricht	22	3	5	14	14	46
Nijmegen	39	19	39	26	122	94
Rotterdam	26	16	16	32	69	72
Utrecht-0.4	45	20	39	48	87	101
Average[3]	31	12	25	35	77	75
P50	27	13	26	37	81	77
P80	39	19	39	48	95	94

[1] Calculation of RBA: $AUC_{soil}/dose_{soil}$ divided by $AUC_{PbAc}/dose_{PbAc}$. The data for PbAc were calculated with the dose-response curve obtained in the pilot study (AUC = 0.36 x dose – 24.2)
[2] Ratio between lead concentrations in extractions with diluted HNO_3 and *aqua regia*
[3] The figures for the *in vitro* models excluding the samples from The Hague. When including The Hague the values are almost the same (max. difference of 4%), except for HNO_3/aqua regia: with The Hague, the average=69, P50=72 and P80= 89

The bioavailability as derived by the different methods can be ordered as follows:
0.43 M HNO_3 ~ $UBM_{gastric}$ > IVD ~ *in vivo* ~ UBM > Tiny-TIM.

8.3.2 *Best applicable in vitro model*

The aim of the present study was to select the model showing the highest correlation with the *in vivo* data as the model to be used in practice, in order to predict the bioavailability of lead in made grounds in young children. Wragg et al. (2011) gave criteria for the linear relationship between the relative bioaccessibility and the relative bioavailability: R^2 of correlation should be higher than 0.6 and the slope should between 0.8 and 1.2. Further criteria for the best model are: the selected model should be simple, responsive to different lead and soil characteristics, and accompanied by rigorous Quality Assurance/Quality Control data requirements (Hagens et al. 2009). In addition, Wragg et al. (2011) mention that the model should be physiologically based and conservative (it should not underestimate the bioavailability). Note that for the aim of the present study it is more important that the model predicts the bioavailability well, rather than conservatively. In Table 20 the criteria are given in order of importance (the most important at the top) and scored for each model. Below the *in vitro* models are discussed with respect to these criteria.

Table 20. Performance of the in vitro models and diluted HNO_3 extraction with respect to criteria for best applicable model

Criterion	Tiny-TIM	IVD	$UBM_{gastric}$	UBM	HNO_3
Correlation (R^2 > 0.6)	+	-/+[1]	-	+	+
Correlations (0.8 < slope < 1.2)	-	-	-	+(/-)[2]	-
Simplicity	-	+	+	+	++
QA/QC	+	-/+[3]	+	+	+
Conservativeness/goodness of prediction	-	+	+	+	+
Responsive to Pb/soil characteristics	+	+	+	+	+
Physiologically based	++	+	+	+	-
Simulation of semi-fed child	+	+	-	-	-

[1] Only when corrected for fraction of calcite in the soil a good correlation is obtained.
[2] Slope equals 1.21
[3] Reproducibility of BAc of PbAc is low.

The range of the determined bioavailabilities of the tested soils is only a factor two and for this reason a correlation is relatively difficult to identify. However, a high correlation can still give an indication of the good performance of an *in vitro* model. As mentioned above, of the three *in vitro* models tested, the UBM gives the best correlation (R^2 = 0.80), with the Tiny-TIM model (R^2 = 0.67) just behind. The IVD model only correlates well after a modeled correction for calcite. However, as this correction is only based on six soils and the scientific rationale is lacking, this is not considered a robust approach. Possibly, if 0.06 g of soil was used in the IVD model, the effect of calcite would be avoided. For this reason, further investigation is recommended.
The correlation between $UBM_{gastric}$ and *in vivo* data is low. In contrast, the lead concentration in the diluted HNO_3 extracts correlates well with the *in vivo* RBA values (R^2 = 0.73).
The slope of the relation between UBM and the *in vivo* RBAs is 1.21. This is very close to the critical value for a validated model (Wragg et al. (2011). For Tiny-TIM the slope equals 0.57, which is lower than the critical value.

Although the Tiny-TIM model shows a fair correlation with the *in vivo* data, the model does not meet the criterion that location-specific determinations should be simple. Furthermore, the RBAs and the BAs of the Tiny-TIM model are lower than the *in vivo* data due to the filtration step, and for this reason they are neither conservative nor realistic.

UBM has a good correlation with the *in vivo* data, is relatively simple, and predicts the *in vivo* data well, although it simulates fasted rather than semi-fed conditions, and uses a solid: liquid ratio of 1:100 which is higher than the ratio of 1:1000 estimated for hand-to-mouth behavior (Bakker and Hagens, 2010). While fasted conditions lead to a higher bioaccessibility than semi-fed conditions due to a lower pH, the solid: liquid ratio of 1:100 does the opposite, due to saturation effects (Hagens et al. 2009). It seems that the net effect of these two factors result in a RBAc comparable to the *in vivo* data.

In general, the quality assurance/ quality control data of the *in vitro* models (i.e. results of the blanks, the reproducibility of the tests using Montana soil and lead acetate) indicated that they produced reproducible and reliable results. There is one exception, however: the bioaccessibility of lead acetate of the IVD model was not reproducible. Since the cause of this is unknown, this needs further investigation.

The main criterion not met by the diluted HNO_3 extraction is that it is not physiologically based. Apparently, this simple and cheap extraction method is a good predictor of the bioavailability of lead from made grounds. As this extraction was only tested for these six soils, and is neither physiologically based nor validated for made grounds before, it is suggested it may at present only be used as a screening method. Nevertheless, this is a promising approach and it is worthwhile to investigate its use for more than screening purposes.

In summary, none of the models meet all the criteria. Nevertheless, the UBM model comes close, since it only fails the least important criterion, namely to mimic a semi-fed child. Apparently, the combination of fasted conditions (increasing bioavailability) and 0.6 g of soil (decreasing bioavailability) yields RBAs comparable to *in vivo* RBAs for the six soils tested. This implies that at present, the UBM model is the best applicable model for the determination of location-specific bioaccessibility of lead in made grounds. As it is known that an amount of 0.6 g reduces the bioaccessibility of lead, it is recommend to further test the six soils with UBM (in a semi-fed state), and with IVD as well, using 0.06 g rather than higher amounts of soil.
The second best applicable 'model' is the extraction with diluted HNO_3. Further investigation of this method is recommended by literature research into the use of this solvent in ecological risk assessment, and by comparison of its results to (validated) physiologically based models for a large number of soils.

8.3.3 Use of PPS-ranking

As shown in Chapter 7, PPS-ranking gives an indication of bioavailability of lead (higher rank predicting a higher bioaccessibility). In the present study, five soils were given a PPS rank, and from Figure A11 in Appendix 6 it can be concluded that the ranking has a fair correlation with the RBA values determined in the blood of the swine. This implies that when information on lead and soil characteristics is available for a soil sample, an indication of the bioavailability of lead of this soil can be given. However, in general, the available information on

the lead characteristics will be the only source of the lead and not the isotope ratio or imaging of the lead-containing particles.

8.3.4 Derivation of Rel F

Although the bioavailability of each soil can in principle be estimated with *in vitro* models, a less specific but efficient way is to derive a generic value for all Dutch made grounds. Based on 1) this generic factor and 2) information on the lead concentration in soil, the bioavailability of lead of a specific location can be estimated. At present, the use of a generic factor to take into account the bioavailability of lead in made grounds has already been made available in the Dutch legislation (Staatscourant, 2012). Since the *in vivo* bioavailability data from the present study differ by only a factor of about two for the six soils, this confirms that a generic bioavailability factor indeed may be suitable, and that the currently used value can be scientifically evaluated with the data from the present study.

The generic factor as presently applied in Dutch legislation and in Sanscrit (the model used for risk assessment of substances in soil, www.sanscrit.nl) is the *Rel F*, the ratio of the bioavailable fraction of lead in the soil and the bioavailable fraction of lead from the diet:

$$Rel\ \mathrm{F}_{\mathrm{lead}} = \frac{\mathrm{F}_{\mathrm{lead\ from\ soil}}}{\mathrm{F}_{\mathrm{dietary\ lead}}} \qquad [8]$$

Where F = bioavailable fraction and *Rel* stands for relative; $F_{lead\ from\ soil}$ is the bioavailable fraction of lead from soil and $F_{dietary\ lead}$ is the bioavailable fraction of lead in the diet. The current value for Rel F for lead in made grounds in the present legislation, and in Sanscrit (the computer program to estimate risks from substances in soil) is 0.4. This is an average of the Rel F values determined for the IVD and Tiny-TIM model in the study of Hagens et al. (2009).

Rel F equals the RBA measured in our *in vivo* study, in which lead acetate in a ball of feed can be considered dietary lead. To determine the *Rel F* from the *in vivo* study with juvenile swine, the relative bioavailabilities of the six soils (expressed as a fraction rather than as a percentage) are taken from Table 13 and presented in Table 21.

Table 21. Rel F for lead in six made grounds determined in the in vivo study (Rel F equals RBA expressed as a fraction)

Value	Rel F
Minimum	0.47
P50	0.58
Average	0.66
P80	0.84
Maximum	0.95

On average, the lead present in made grounds is 66% available for uptake compared to lead from the diet, while the maximum value is 95%. In other words, the lead in made grounds has, on average, a lower bioavailability than dietary lead, but still can be as bioavailable as dietary lead. For this reason, it is clear that the current value for *Rel F* of 0.4 is too low from a scientific point of view and for this reason it is recommended to increase the value to e.g. 0.58

(P50) - 0.84 (P80), depending on the chosen level of conservatism. This range corresponds with the Rel F determined for 90 soils in the previous study (Hagens et al. 2009), which equaled 0.67 (P50) – 0.91 (P80).

Since the variation found in the bioavailabilities of lead in made grounds is only a factor of two, this finding implies that the role of location-specific determinations of bioaccessibility of lead may be small. The reason is that the likelihood that a soil sample has a significantly lower bioavailability than the generic value is small. For example, from the samples in this study, only Maastricht has a Rel F significantly lower than 0.58 (namely 0.47; locations Rotterdam, De Rijp en Leiden have values around 57%). Also, of the 90 samples tested by Hagens et al. and determined with IVD (0.06 g), ten soils showed a Rel F < 0.5 and only three were smaller than 0.4.

8.4 Variability and uncertainty

Variability
The main sources of variability were the test soils sampled in this study: the experimental variability in the pilot *in vitro* experiments, using milled soil samples from the previous study, was low and this was also the case for the results of the Montana soil. In the validation experiment, the overall RSD of the RBAs was within 20%, but for some soils (Nijmegen, Maastricht and The Hague) the RSD in the *in vitro* models was high (up to 55%). This high RSD was likely due to heterogeneous soil samples, which particularly affected the *in vitro* results, as in these experiments only a small amount of soil is used compared to amount of soil given to the swine in the *in vivo* study. Although care was taken to homogenize the soil samples thoroughly, including an additional 'splitting' step, apparently the homogenization was incomplete. This can also be concluded from 1) recoveries in Tiny-TIM were sometimes unequal to ~100% and 2) the total lead concentrations of the Maastricht subsample were lower with XRF than those obtained with extractions with *aqua regia.* Nevertheless, also with the heterogeneous soil samples the correlations between *in vivo* data and the *in vitro* models UBM and Tiny-TIM were strong.
The variability in the swine was acceptable: on average the RSD in the *in vivo* RBAs was 21%, while the highest RSD equaled 33%.

Uncertainty
The results on lead acetate in the validation study, both for the IVD model and the *in vivo* study can be considered unreliable. An explanation for these findings cannot be given. For this reason, the RBA values of both the *in vivo* experiment and the IVD model are considered relatively uncertain as they were calculated using data on lead acetate from their respective pilot experiments. The uncertainty in these data influences the magnitude of the RBA values (and therefore the magnitude of the Rel F). Nevertheless, it does not affect the correlations determined between the different models.
Additionally, the selected value for the bioavailability of dietary lead in children (47%) is uncertain, which affects the ABA in children.

9 Conclusions and recommendations

9.1 Conclusions

Although the bioavailability of the orally dosed lead acetate was low in the *in vivo* study, relative bioavailability values for the six soils could be established by using the lead acetate bioavailability from the pilot study.

Strictly spoken, none of the *in vitro* models meet the set criteria for a validated model for the bioavailability of lead from made grounds. Nevertheless, the UBM model only fails one, less important, criterion, since it does not simulate a semi-fed child. UBM appears to be the best applicable model for use in location-specific determination of the bioaccessibility of lead in made grounds.

Comparable with UBM, Tiny-TIM also showed a high correlation with the *in vivo* RBAs, but this model resulted in lower bioavailability values than the *in vivo* study. Results of the IVD model did not correlate with the *in vivo* data. Only when using a correction for calcite content of the soil, can the IVD model predict the bioavailability of lead well. However, as this correction is only based on six soils and the scientific rationale is lacking, this is not considered a robust approach.

Extractions with diluted HNO_3, although not physiologically based, correlate well with the *in vivo* results on bioavailability of lead in made grounds and are conservative with respect to the six tested soils (i.e. give higher bioaccessibility than *in vivo*). For these reasons, this extraction method may be used as a first screening method, and perhaps as a prediction method for bioavailability, once it has been further tested and validated.

Based on the values from the *in vivo* study, a generic bioavailability factor for made grounds has been deducted which can be applied in human risk assessment of lead in inner cities. The results indicate that the bioavailability of lead in made grounds can be as large as the bioavailability of dietary lead. Based on these results (n=6 soils) it can be concluded that the present *Rel F* of 0.4 is too low from a scientific point of view and that the Rel F should be increased to a value in the range of 0.58 (P50)– 0.84 (P80), taking into account the desired level of conservatism.

Due to the small variation in the RBAs of the soils, the role of location-specific determination of bioaccessibility of lead in made grounds is expected to be small.

9.2 Recommendations for research

- If, for bioaccessibility testing of lead from soils, an *in vitro* model is desired which simulates a semi-fed child, validation of UBM and IVD under semi-fed conditions with the six test soils using 0.06 g soil ($n \geq 8$) is recommended.
- Validate the extraction method with diluted HNO_3, with additional animal experiments, by performing a literature search into correlations between diluted HNO_3 extractions and bioavailability/bioaccessibility data, and/or by generating these data (determine bioaccessibility with HNO_3 and validated soils and/or *in vitro* method).

- Determine the bioavailability of dietary lead acetate in an additional experiment with juvenile swine, in order to establish a more reliable *in vivo* RBA, and therefore a more trustworthy *Rel F*.

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Appendix 1 In vitro digestion model under semi-fed conditions

In vitro digestion model under semi-fed conditions

The IVD model under semi-fed conditions was based on the IVD model for fasted conditions and the IVD model under fed conditions (Oomen et al., 2003; Versantvoort et al., 2005). Soil samples (dry weight) were weighed and used with or without supplementation of 1 g of standard Dutch dinner (Olvera 15M52 with 2% (w:w) sunflower oil). A volume of 9 ml saliva (pH 6.7) was added to the soil mixture and rotated for 5 min at 37°C. Subsequently, 18 ml gastric juice was added and the pH of the mixture was set to 2.0 ± 0.2. The mixture was rotated for 2 h at 37°C. Finally, 18 ml duodenal juice, 9 ml bile juice and 3 ml sodium carbonate solution were added and the pH of the mixture (digestion juice) was set to 6.3 ± 0.2. The mixture was rotated for 2 h at 37°C, centrifuged for 5 min at 2900 g, diluted 1: 100 with 0.1 M HNO_3 and stored at -20°C until analysis. For ultrafiltration, the chyme samples (0.2 g soil, location Leiden, standard model) were centrifuged at 1000 g for 5 min. The supernatant was transferred to a 10 kDa filter column (Millipore) and centrifuged at 3000g for 60 min at 20°C. In the upscaled model, the amounts were 0.2 g soil, 30 ml saliva, 60 ml gastric juice, 60 ml duodenal juice, 30 ml bile juice, and 10 ml sodium carbonate (see Table A1 for composition of the juices).
Reference soil (Montana 2710a) and a blank (no soil) were included. Furthermore, the bioaccessibility of lead from a spiked lead acetate solution resulting in 0.05 and 5 mg lead in the digestion (with and without food) was investigated in duplicate and used as a reference.

Table A1. Composition of the juices for semi-fed in vitro digestion models (amounts based on 1000 ml juice).

Saliva	Gastric juice
• 896 mg KCl • 200 mg KSCN • 1021 mg $NaH_2PO_4 \cdot H_2O$ • 570 mg Na_2SO_4 • 298 mg NaCl • 1.8 ml 1M NaOH • 200 mg urea • 145 mg amylase • 15 mg uric acid • 50 mg mucin • milli-Q water pH: 6.5 ± 0.1.	• 2752 mg NaCl • 306 mg $NaH_2PO_4 \cdot H_2O$ • 824 mg KCl • 302 mg $CaCl_2$ • 306 mg NH_4Cl • 7.4 ml 37% HCl • 650 mg glucose • 20 mg glucuronic acid • 85 mg urea • 330 mg glucosaminehydrochloride • 1 g BSA • 1.75 g pepsin • 3 g mucin • milli-Q water pH: 1.2 ± 0.1

Duodenal juice	Bile juice
• 7012 mg NaCl • 3388 mg $NaHCO_3$ • 80 mg KH_2PO_4 • 564 mg KCl • 50 mg $MgCl_2 \cdot 6H_2O$ • 180 µl HCl (37%) • 100 mg urea • 151 mg $CaCl_2$ • 1 g BSA • 6 g pancreatin • 1 g lipase • milli-Q water pH: 8.0 ± 0.1.	• 5259 mg NaCl • 5785 mg $NaHCO_3$ • 376 mg KCl • 175 µl HCl (37%) • 250 mg urea • 167.5 mg $CaCl_2$ • 1.8 g BSA • 18 g bile • milli-Q water pH: 8.1 ± 0.1.

Sodium carbonate solution
• 42.3 g $NBaHCO_3$ • milli-Q water

pH adjustments	
The pH of each of the juices is adjusted to the correct pH with NaOH (1 M) or HCl (37%). In addition, the pH of the total digestion juice is measured (1 ml saliva, 2 ml gastric juice, 2 ml duodenal juice, 1 ml bile juice and 14 mg $NaHCO_3$). The pH has to be 6.3 ± 0.2	.

Appendix 2 Extraction of lead from *in vivo* samples

One ml of whole blood was added to 9.0 mL of matrix modifier. The matrix modifier consisted of 1% (v/v) HNO_3 and 0.1% (v/v) Triton X-100.
The femur was placed in a beaker with 2 N NaOH and allowed to soak overnight to digest residual soft tissue. The femur was washed overnight with HNO_2 and dried at 100°C overnight. The dried bones are then broken in half, placed in a muffle furnace, and dry-ashed at 450°C for 48 h. The bone ash is ground in a mortar and a 200 mg portion is dissolved in 10 mL of 1:1 nitric acid (v:v) in water.
One gram of soft tissue (liver or kidney) was placed in a Teflon container with 2 mL of 70% HNO_3 and heated overnight at 90°C. After cooling, the digestate was transferred to a clean 10 mL volumetric flask and diluted to 10 mL with deionized, double distilled water.
Food samples were completely homogenized by mixing in a blender. A sample of 0.25 g was mixed with 4 ml HNO_3 (65%) and 4 ml H_2O_2 (30%). The samples were destructed by heating at 210°C for 85 min. After cooling, solutions were filtered and made up to 25 ml with ultrapure water.

Appendix 3 RBA and RBAc based on XRF analyses

Table A2. Relative bioaccessibilities from IVD model based on lead concentrations in soils as determined by XRF

Sample	Amount soil (g)	Lead intake (µg)	BAc chyme (%)	RBAc chyme (%)[a]
The Hague	0.2	133	44	**74** ± 42
De Rijp	0.2	274	50	**85** ± 7
Leiden	0.2	141	39	**66** ± 5
Maastricht	0.2	198	18	**31** ± 25
Nijmegen	0.2	861	20	**34** ± 45
Rotterdam	0.2	465	37	**62** ± 9
Utrecht	0.2	713	48	**82** ± 6
PbAc	-		59	-

[a] Bioaccessibility of lead from soil relative to bioaccessibility from PbAc as determined in the pilot study.

Table A3. Relative bioaccessibilities from Tiny-TIM based on lead concentrations in soils as determined by XRF

Sample	Amount soil (g)	Lead intake (µg)	BAc dialysate (%)	RBAc dialysate (%)
The Hague	0.5	331	12, 17	**27, 36**
De Rijp	0.5	685	8, 7	**16, 17**
Leiden	0.5	353	5, 4	**9, 11**
Maastricht	0.5	496	3, 4	**7, 8**
Nijmegen	0.5	2143	8, 15	**18, 32**
Rotterdam	0.5	1159	15, 14	**33, 30**
Utrecht	0.5	1784	17, 15	**36, 33**
PbAc	-	1000	47, 46	-

Table A4. Relative bioaccessibilities from UBM based on lead concentrations in soils as determined by XRF.

Sample	Lead intake (µg)[a]	BAc (%)	RBAc[b] (%)± RSD	BAc (%)	RBAc[b] (%)± RSD
		Gastric		Gastric + intestine	
The Hague	662	80	81 ± 3	27	41 ± 19
De Rijp	1370	78	79 ± 4	21	32 ± 8
Leiden	706	88	89 ± 5	29	44 ± 18
Maastricht	991	17	18 ± 7	7	10 ± 29
Nijmegen	4285	92	93 ± 1	29	44 ± 55
Rotterdam	2317	79	79 ± 3	19	28 ± 3
Utrecht	3567	86	87 ± 7	39	59 ± 7
PbAc[c]		99		66	

[a] Based on XRF

[b] Average bioaccessibility relative to PbAc

[c] Bioaccessibility of PbAc was obtained from previous experiments (Denys et al., 2012)

Table A5. Relative bioaccessibilities from in vivo study based on lead concentrations in soils as determined by XRF

Location	RBA based on valid.study[1] (%)	RBA based on pilot study[2] (%)
De Rijp	**86**	**48**
Leiden	**78**	**43**
Maastricht	**88**	**49**
Nijmegen	**90**	**50**
Rotterdam	**91**	**51**
Utrecht-0.4	**136**	**76**
Utrecht - 1	**68**	**38**
PbAc (oral)[4]	**7**	**12**

Table A6. Overview of RBAs and RBAcs from in vivo and in vitro studies

Location	RBA blood	IVD	Tiny-TIM	$UBM_{gastric}$	UBM
The Hague	nd	74	31	81	41
De Rijp	48	85	17	79	32
Leiden	43	66	10	89	44
Maastricht	49	31	7	18	10
Nijmegen	50	34	25	93	44
Rotterdam	51	62	32	79	28
Utrecht	76	82	34	87	59

nd = not detected

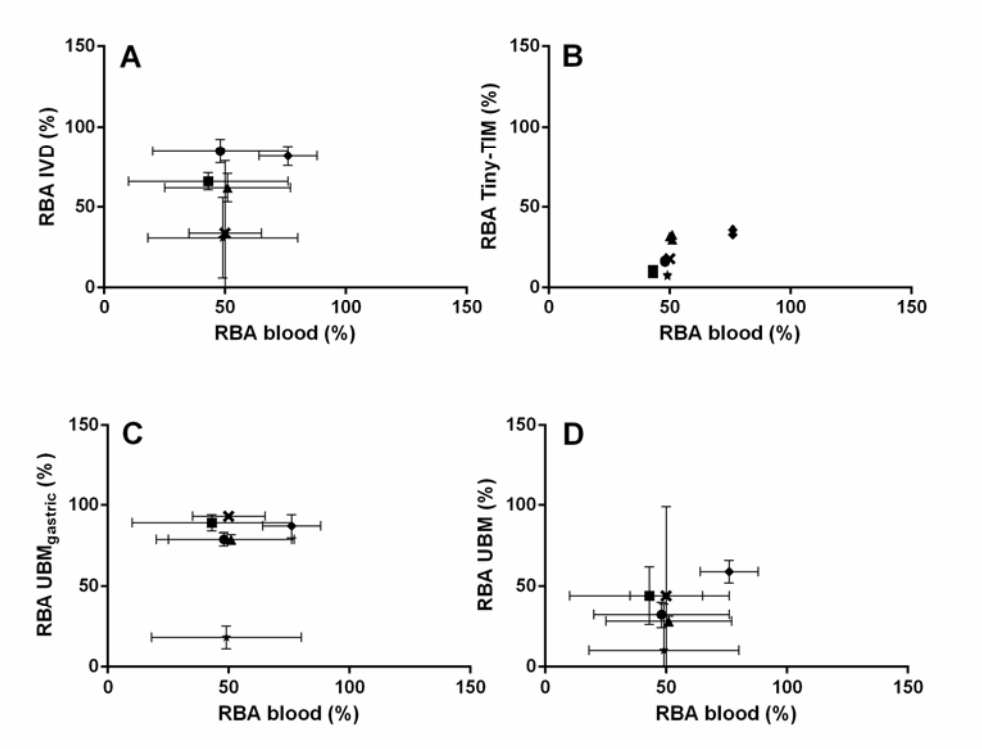

Figure A1. Correlation of the RBA from blood with the RBAc from a) IVD, b) Tiny-TIM system, c) UBM gastric phase and d) UBM gastric + intestine phase, based on lead concentrations in soil as determined by XRF. Experiments with Tiny-TIM were performed in duplicate; both values are presented.

Appendix 4 Plots of single linear regression analyses

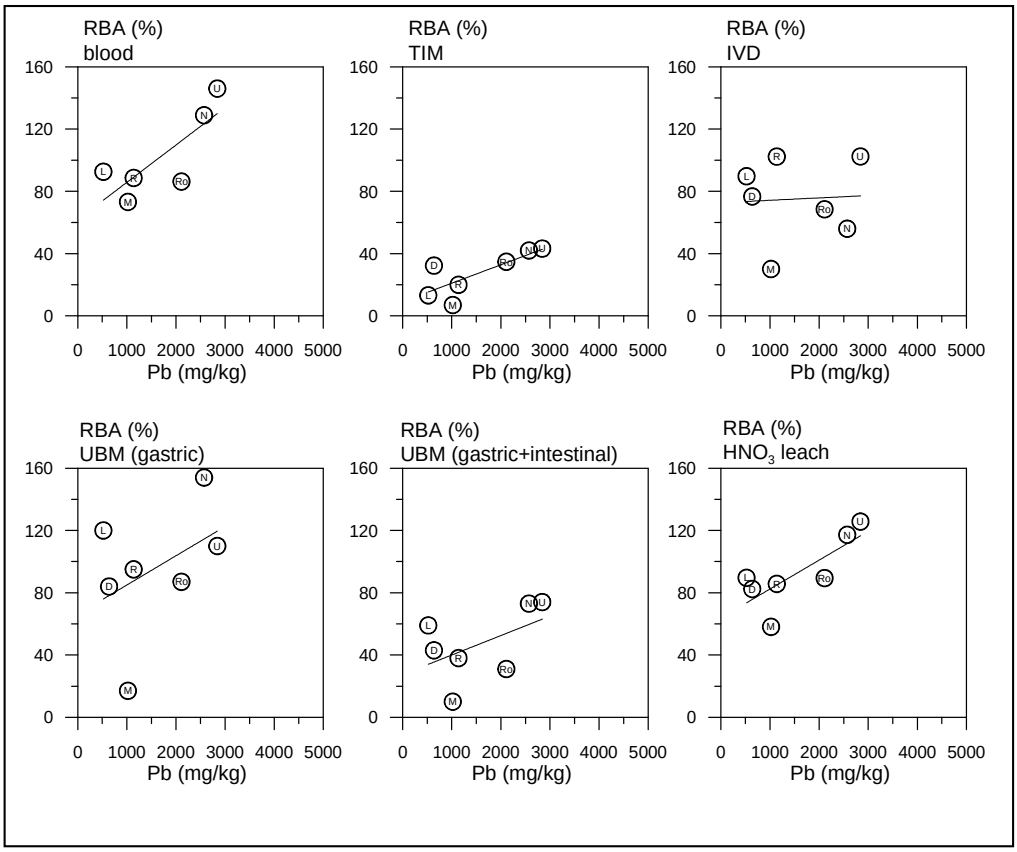

Figure A4. The relative oral bioavailability (RBA) of lead (aqua regia) as determined in the in vivo study (blood pigs) and the in vitro digestion models (Tiny-TIM, IVD, UBM and HNO_3 leach) with the soil characteristic Pb content (aqua regia digestion). In these plots, also a "best fit" trend line is added. Sample locations are: D=The Hague, L=Leiden, M=Maastricht, N=Nijmegen, R=De Rijp, Ro=Rotterdam, U=Utrecht.

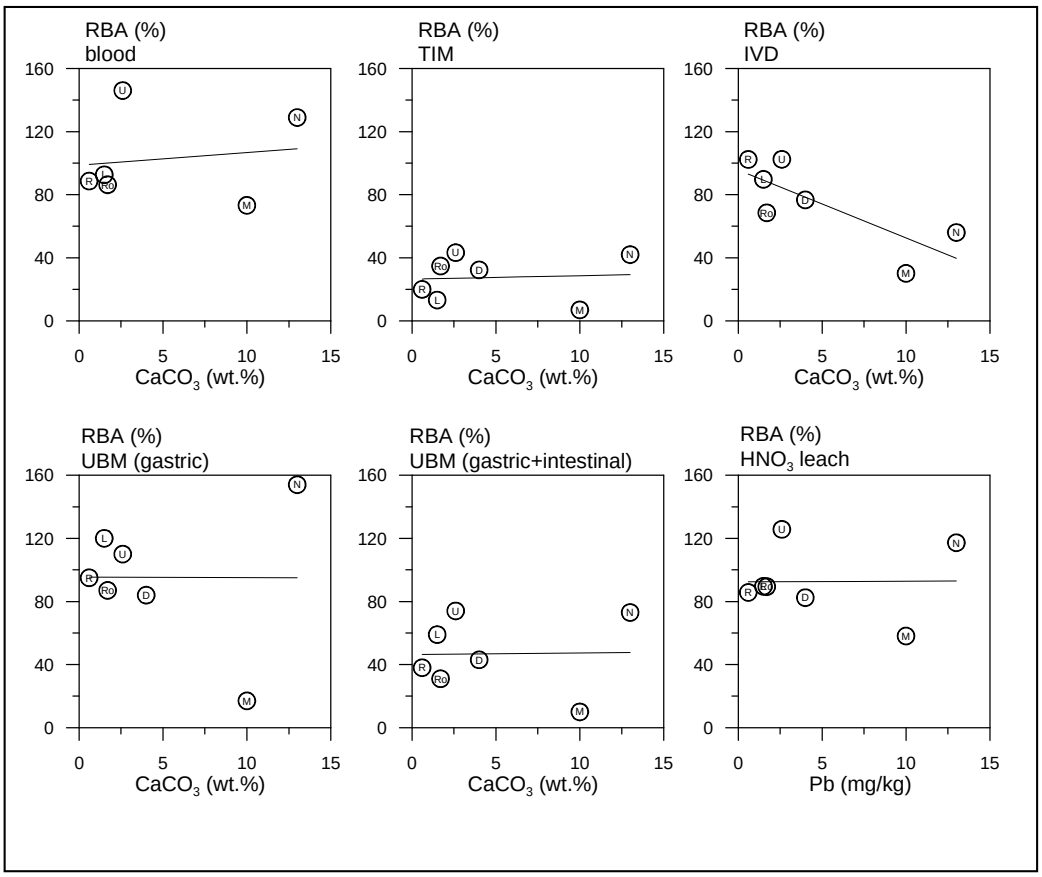

Figure A5. The relative oral bioavailability (RBA) of lead (aqua regia) as determined in the in-vivo study (blood and the in vitro digestion models (TIM, IVD, UBM and HNO_3 leach) with the soil characteristic calcium carbonate content. In these plots, also a "best fit" trend line is added. Sample locations are: D=The Hague, L=Leiden, M=Maastricht, N=Nijmegen, R=De Rijp, Ro=Rotterdam, U=Utrecht

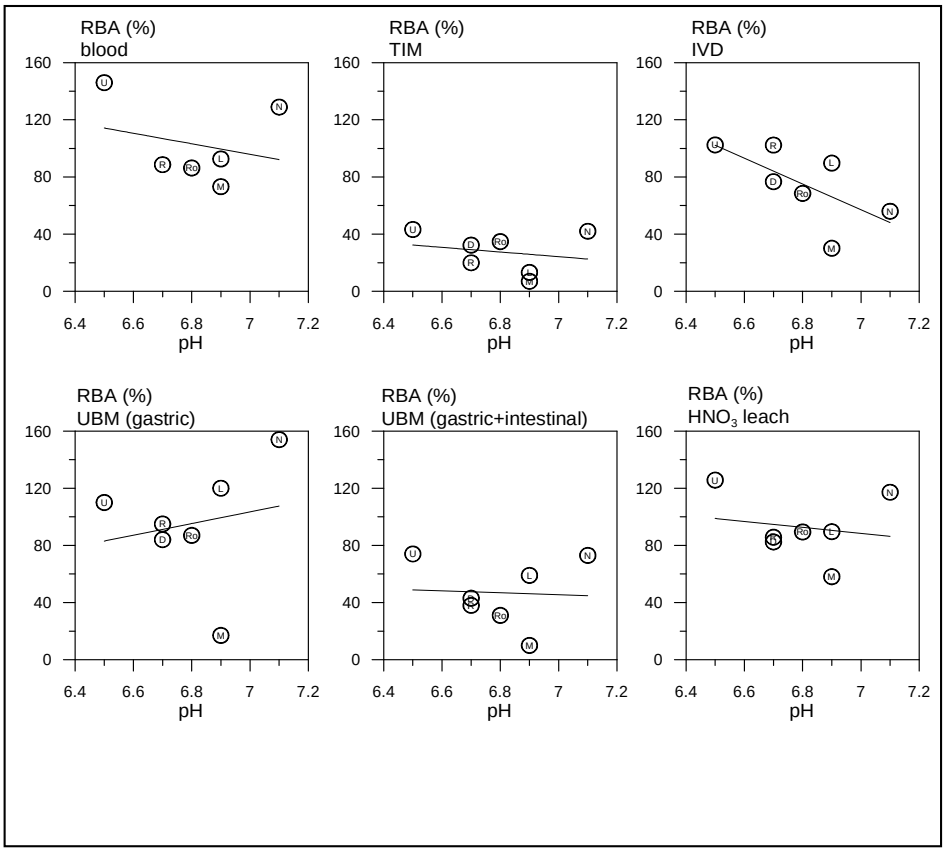

Figure A6. The relative oral bioavailability (RBA) of lead (aqua regia) as determined in the in vivo study (blood pigs) and the in vitro digestion models (Tiny-TIM, IVD, UBM, HNO_3 leach) with the soil characteristic soil pH. In these plots, also a "best fit" trend line is added. Sample locations are: D=The Hague, L=Leiden, M=Maastricht, N=Nijmegen, R=De Rijp, Ro=Rotterdam, U=Utrecht.

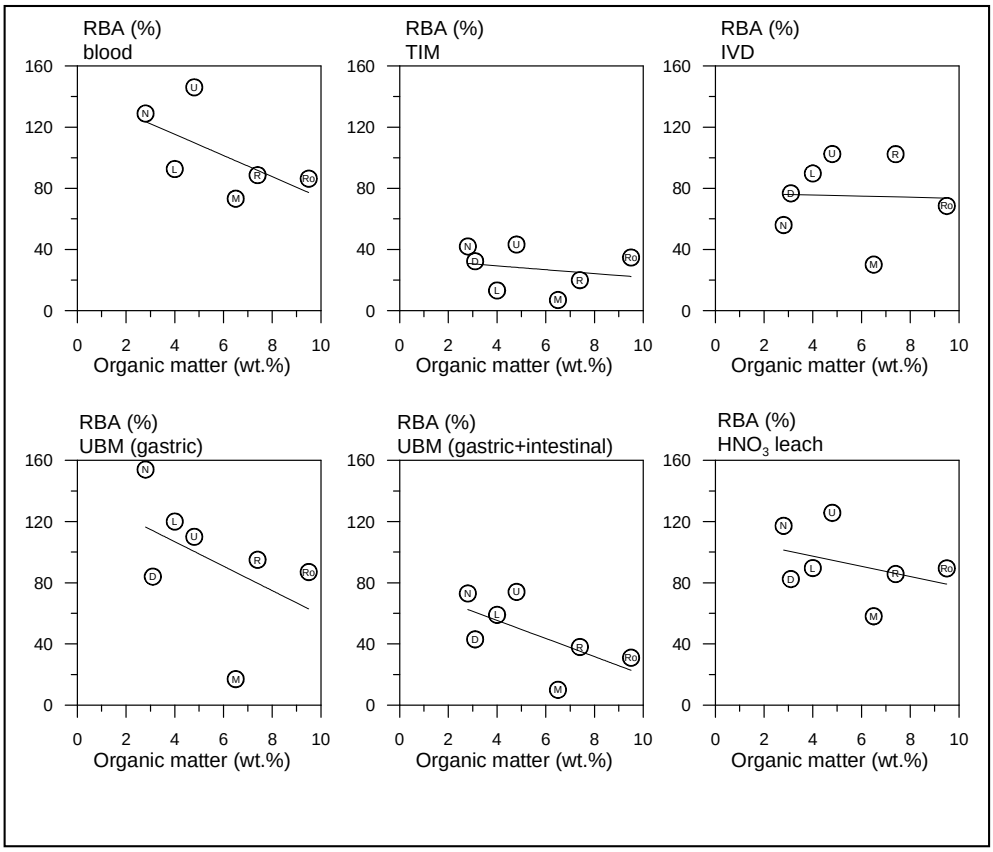

Figure A7. The relative oral bioavailability (RBA) of lead (aqua regia) as determined in the in vivo study (blood) and the in vitro digestion models (Tiny-TIM, IVD, UBM and HNO_3 leach) with the soil characteristic organic matter content. In these plots, also a "best fit" trend line is added. Sample locations are: D=The Hague, L=Leiden, M=Maastricht, N=Nijmegen, R=De Rijp, Ro=Rotterdam, U=Utrecht.

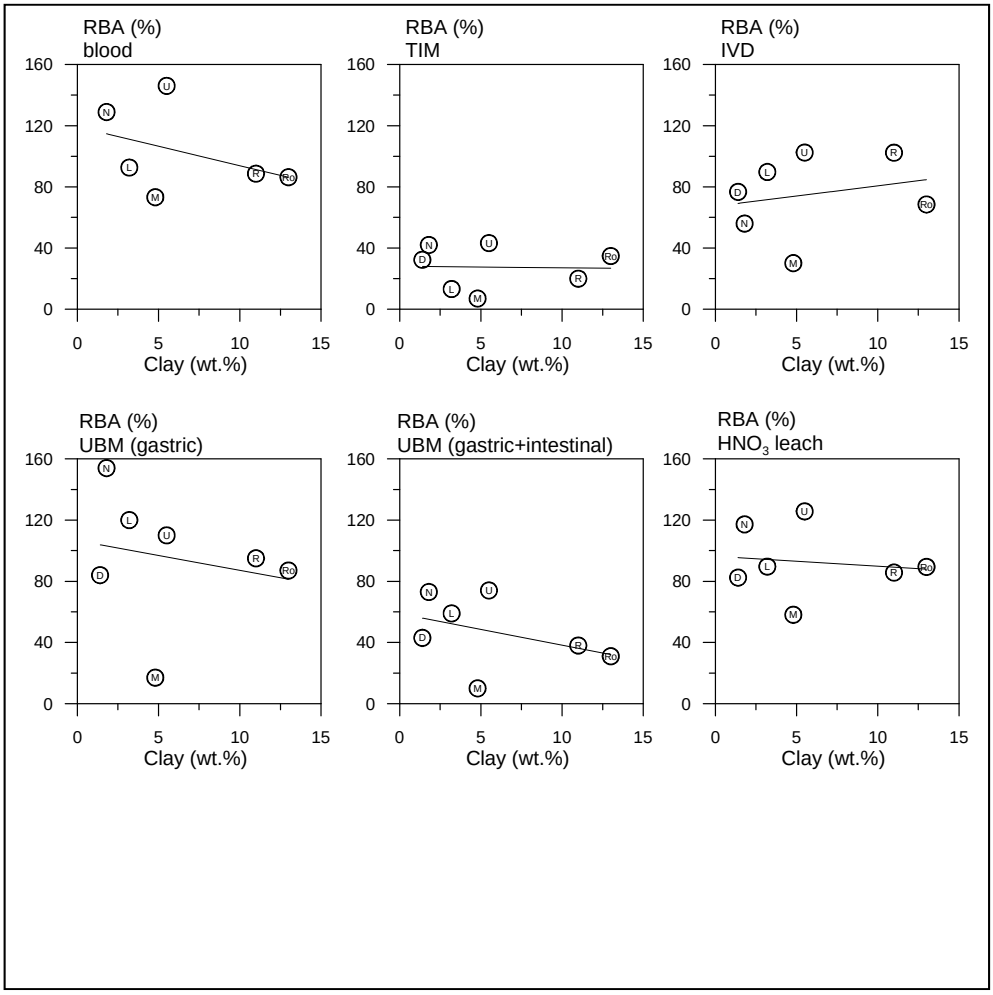

Figure A8. The relative oral bioavailability (RBA) of lead (aqua regia) as determined in the in vivo study (blood pigs) and the in vitro digestion models (Tiny-TIM, IVD, UBM and HNO_3 leach) with the soil characteristic clay content. In these plots, also a "best fit" trend line is added. Sample locations are: D=The Hague, L=Leiden, M=Maastricht, N=Nijmegen, R=De Rijp, Ro=Rotterdam, U=Utrecht.

Appendix 5 Plots of multiple regression analyses

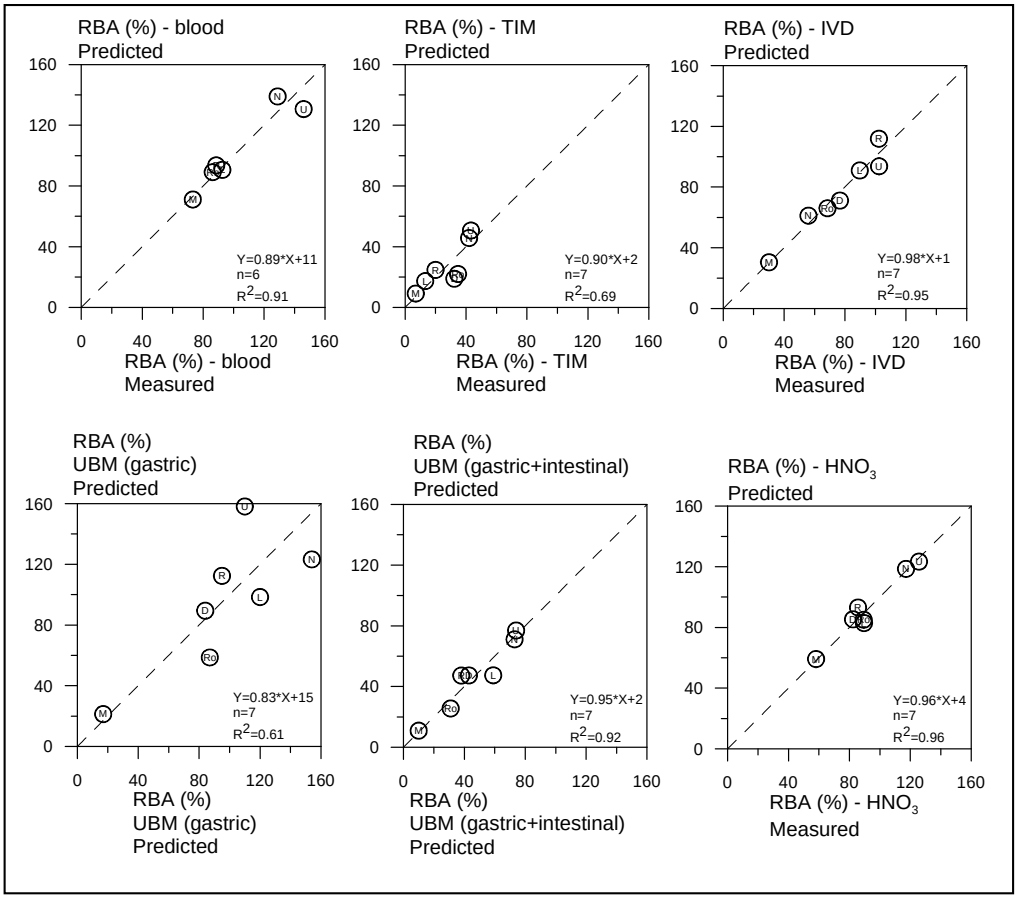

Figure A9. Measured relative oral bioavailability of lead (expressed as %) versus the predicted relative oral bioavailability of lead (expressed as %). The solid line represents the 1:1 line. Sample locations are: D=The Hague, L=Leiden, M=Maastricht, N=Nijmegen, R=De Rijp, Ro=Rotterdam, U=Utrecht.

Figure A9 shows that the fraction of variance explained by the model varies from 61% with the UBM (gastric) model to 96% with the HNO_3 leach (= Multiple R-squared).

Appendix 6 Influence of lead characteristics on RBA

For the description of lead characteristics, primary lead phases are distinguished from secondary lead phases. Primary lead phases are the lead containing particles that entered the soil (e.g. lead glass, elemental lead (bullet) and white lead (paint)). Two groups of primary lead phases are distinguished:

1) Elemental lead, lead oxide and/or lead carbonate. These phases are characterized by a very high lead content (> 90 wt %) (for SEM photos see Appendix 7)
2) Lead glass or lead glaze. Lead glass is characterized by a high SiO_2 and lead content. Lead glaze is often present on clay (ceramic) and is therefore characterized by a high Al_2O_3, SiO_2 and lead content (see Appendix 7 for SEM photos).

The samples from De Rijp, Leiden, and Maastricht are mainly polluted with the primary lead phases lead glass and/or lead glaze. The diameter of these phases is relatively large. The sample from Nijmegen is mainly polluted with elemental lead, lead oxide and/or lead carbonate. The diameter of these primary lead phases is relatively small. Since the sample from Utrecht was taken from the close vicinity of a former lead white factory, it most likely contains elemental lead, lead oxide and/or lead carbonate (with a small diameter). The primary lead phases in the samples from The Hague and Rotterdam are unknown.

Over time, primary lead phases in the soil can dissolve and secondary lead phases can be formed. In total, four secondary lead phases are distinguished:

1) Lead apatite[5].
2) Pb-OM: Lead adsorbed to organic matter (OM).
3) Fe-Pb: Lead adsorbed to reactive iron.
4) Pb-S: Lead sulphate or lead sulfide (galenite). This phase can also have a primary nature.

Lead apatite minerals are detected in the made grounds from De Rijp, Leiden and Nijmegen (See Appendix 7 for a SEM analysis of a lead apatite mineral). In all four samples (De Rijp, Leiden, Nijmegen, Maastricht), lead was found to be bound to organic matter (Table A7). However, the lead content of organic matter particles in soils from De Rijp, Leiden and Maastricht is very low (Table A7). Pb-Fe and Pb-S phases were not detected in the samples.

[5] Minerals belonging to the apatite group have the following general formula $A_5(XO_4)_3$-(F, Cl, OH), where A=Pb, Ba, Ca, Ce, Na and Sr, and X=As, P, Si and V. Lead apatites in made grounds are characterized by high contents of Pb, Ca, P (P_2O_5) and/or Cl. The lead apatite $Pb_5(PO_4)_3Cl$ is known to be very stable (Ruby *et al.* 2000). Lead apatite can be newly formed or formed through adsorption or substitution of lead on already existing apatite (e.g bone flakes).

Table A7. Lead characteristics (FCM cluster labels, lead isotopic compositions, and primary and secondary lead phases, particle size and the mean lead content of organic matter as determined with the SEM) of the made ground samples from De Rijp, Leiden, Nijmegen and Maastricht as determined by Hagens et al. (2009).

Sample location		FCM cluster	Pb isotopes		Primary Pb phases			Secondary Pb phases			
			$^{206}Pb/^{207}Pb$	$^{208}Pb/^{206}Pb$	Pb, Pb oxide Pb carbonate	Pb glass /glaze	Pb-S	Pb-apatite	Pb-organic matter	Pb-S	Pb-Fe
17	De Rijp	2	1,175	2,090	-	++ (20-675 μm)	-	+ (1 particle: 25 μm)	+- [n=19: 0.020]	-	-
29	Leiden	3	1,170	2,099	-	+ (5-15 μm)	-	+ (25 μm)	+- [n=11: 0.004]	-	-
63	Nijmegen	4	1,158	2,102	++ (2-195 μm)	-	-	+- (1 particle: 30 μm)	++ [n=12: 0.335]	-	-
71	Maastricht	3	1,161	2,095	+- (1 particle: 5 μm)	+ (10-40 μm)	-	-	-+ [n=23: 0.004]	-	-

- absent; +- few particles present; + several particles present; ++ large number of particles present; () diameter of Pb phase; [] mean Pb content (in wt %) of organic matter particles.

PPS ranking

Each of the lead characteristics (primary phases and particle size) is assigned a value on a 1-4 scale (Table A8). If both lead apatite and lead adsorbed to organic matter (mean lead > 0.05 wt %) are present, the sample is assigned a value of -1 (Table A8). This is done to correct for the higher stability of secondary minerals compared to the primary minerals. The sum of the values constitutes the so-called PPS index, in which the PPS stands for Primary lead phases, Particle size and Secondary lead phases. A PPS index of 1 predicts that lead in a soil samples is not readily bioavailable and a PPS index of 8 predicts that the lead pollution is very bioavailable. It is recognized that the PPS index is arbitrary, but based on the available data, this is the most attainable way to present the data.

Table A8. The PPS ranking system (Primary lead phases, Particle size and Secondary lead phases) (from Hagens et al. (2009)).

Lead characteristic -See also figure 7.1	Category **P** Primary lead phases	 **P** Particle size	 **S** Secondary lead phases
PbS; Pb^0 ; $Pb_5(PO_4)_3Cl$; Pb glass/glaze	**1**		
Fe-Pb oxides ; $PbSO_4$; $PbCrO_4$; Pb phosphate	**2**		
Pb oxides and carbonates (e.g. Pb_3O_4)	**3**		
Pb-halides (e.g. PbCl)	**4**		
Particles (<10 to > 500 µm)		**1**	
Particles (<10 to 500 µm)		**2**	
Particles (<10 to 100 µm)		**3**	
Particles (<15 µm)		**4**	
Secondary Pb-apetite / Pb-Organic Matter			**-1**

In this study, two groups of primary lead phases are distinguished: 1) Elemental lead (Pb^0), lead oxide and/or lead carbonates and 2) lead glass and lead glaze. Group 1 is ranked as category 3 (lead oxides and lead carbonates). Since the sampled soils are mainly aerobic, present elemental lead is most likely oxidized to lead oxides. Group 2 is ranked as category 1. In case both groups of primary lead phases are present in a made ground' sample, the sample is ranked as a category 3 (worst case scenario).

The results of the PPS ranking are listed in Table A9. The PPS ranking varies from 2 to 7. The soil sample from De Rijp revealed the lowest PPS ranking (2). This is due to the presence of large particles of lead glass/glaze. It is predicted that the soil sample form Utrecht will have the highest PPS ranking (7). This is due to the presence of small particles of lead white.

Table A9. The results of the PPS ranking.

Sample location	**P** Primary lead phases	**P** Particle size	**S** Secondary lead phases	**PPS** Ranking
De Rijp	1	1		2
Leiden	1	4		5
Nijmegen	3	2	-1	4
Maastricht	1	3		4
Utrecht	3 (predicted)	4 (predicted)	nd	7
Rotterdam	nd	nd	nd	nd
The Hague	nd	nd	nd	nd

nd= not determined

In Figure A11, the relative oral bioavailability of lead, determined by the *in vivo* and *in vitro* models are plotted versus the PPS ranking. This ranking takes, besides the primary lead phases, also the particle size and presence of secondary lead phases into account.

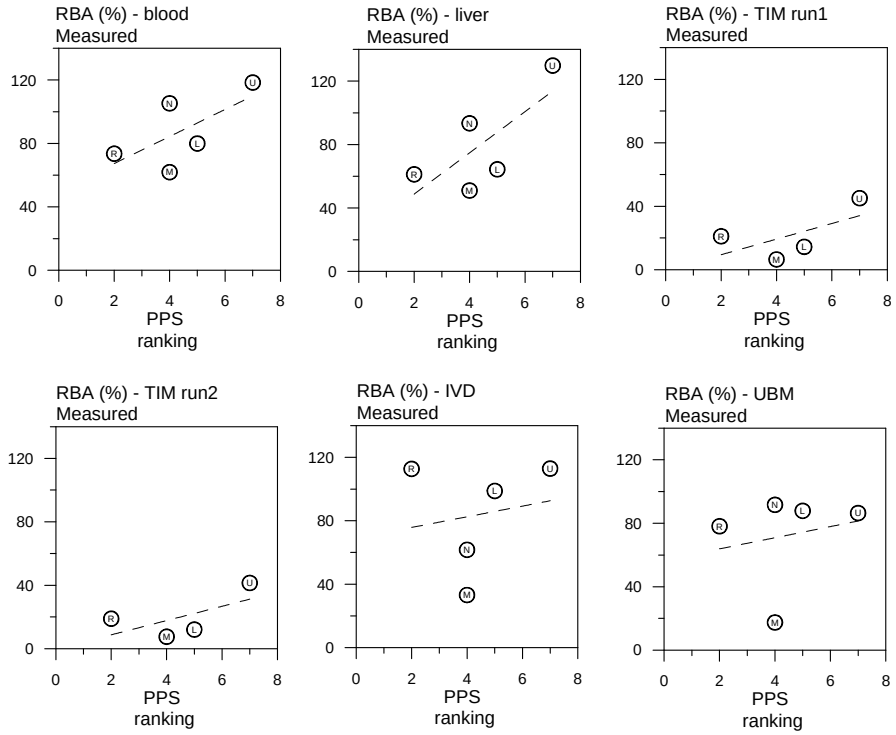

Figure A11. Relative oral bioaccessibility factor for lead (RBA aqua regia) versus the PPS ranking. Sample locations are: D=The Hague, L=Leiden, M=Maastricht, N=Nijmegen, R=De Rijp, Ro=Rotterdam, U=Utrecht.

Appendix 7 SEM photos

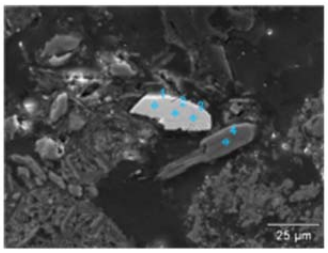

Photo 1. Pb glass/glaze particle in soil sample from Maastricht (77).

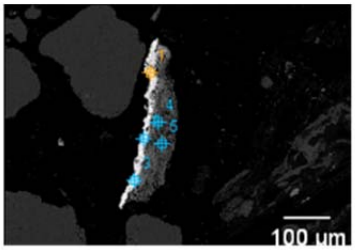

Photo 2. Pb glass/glaze particle (Pb glazed potsherd) in soil sample from De Rijp (17).

Photo 3. Pb oxide in soil sample from Nijmegen (63).

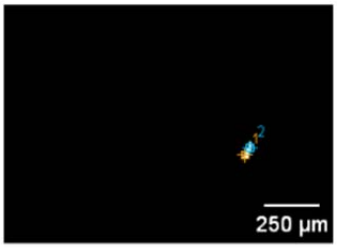

Photo 4 Elemental Pb, Pb oxide or Pb carbonate in soil sample from Haarlem (21).

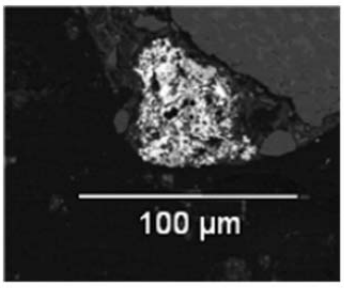

Photo 5. Pb apatite in soil sample from Alkmaar (27).

Photo	Point	Al_2O_3	SiO_2	P	Fe_2O_3	CaO	Pb
1	1	5	29	<1	<1	4	60
2	1, 2	6	34-36	<1	2	2	50-53
3	1	<1	<1	<1	<1	<1	99
4	1, 2	<1	<1	<1	<1	<1	97
5	-	1-2	0-7	17-18	2-3	8-10	57-68

Figure A12. Examples of primary and secondary lead phases, and their chemical composition, in made grounds (source: Hagens et al., 2009).

RIVM

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